

Assays to determine the biological activity of a Type I IFN modified by the present invention will be well known to the skilled artisan. For example, the assay described in Rubinstein et al., (1981, Journal of Virology 37:755-758) is commonly used to determine the effect of an Type I IFN by measuring the cytopathic effects of viral infection on a population 5 of cells. This method is only one of many known in the art for assaying the biological function of a Type IFN.

#### C. Factor VIIa

The present invention further encompasses a method for the remodeling and 10 modification of Factor VII. The blood coagulation pathway is a complex reaction comprising many events. An intermediate event in this pathway is Factor VII, a proenzyme that participates in the extrinsic pathway of blood coagulation by converting (upon its activation to Factor VIIa) Factor X to Xa in the presence of tissue factor and calcium ions. Factor Xa in turn then converts prothrombin to thrombin in the presence of Factor Va, calcium ions and 15 phospholipid. The activation of Factor X to Factor Xa is an event shared by both the intrinsic and extrinsic blood coagulation pathways, and therefore, Factor VIIa can be used for the treatment of patients with deficiencies or inhibitors of Factor VIII. There is also evidence to suggest that Factor VIIa may participate in the intrinsic pathway as well therefore increasing the prominence and importance of the role of Factor VII in blood coagulation.

20 Factor VII is a single-chain glycoprotein with a molecular weight of approximately 50 kDa. In this form, the factor circulates in the blood as an inactive zymogen. Activation of Factor VII to VIIa may be catalyzed by several different plasma proteases, such as Factor XIIa. Activation of Factor VII results in the formation of a heavy chain and a light chain held together by at least one disulfide bond. Further, modified Factor VII molecules that cannot 25 be converted to Factor VIIa have been described, and are useful as anti-coagulation remedies, such as in the case of blood clots, thrombosis, and the like. Given the importance of Factor VII in the blood coagulation pathway, and its use as a treatment for both increased and decreased levels of coagulation, it follows that a molecule that has a longer biological half-life, increased potency, and in general, a therapeutic profile more similar to wild-type Factor 30 VII as it is synthesized and secreted in the healthy human would be beneficial and useful as a treatment for blood coagulation disorders.

Factor VII has been cloned and sequenced, and the nucleic acid and amino acid sequences are presented herein as SEQ ID NO:7 and SEQ ID NO:8 (Figure 55A and 55B, respectively). The present invention should in no way be construed as limited to the Factor VII nucleic acid and amino acid sequences set forth herein. Variants of Factor VII are 5 described in, for example, U.S. Patent Nos. 4,784,950 and 5,580,560, in which lysine-38, lysine-32, arginine-290, arginine-341, isoleucine-42, tyrosine-278, and tyrosine-332 is replaced by a variety of amino acids. Further, U.S. Patent Nos. 5,861,374, 6,039,944, 10 5,833,982, 5,788,965, 6,183,743, 5,997,864, and 5,817,788 describe Factor VII variants that are not cleaved to form Factor VIIa. The skilled artisan will recognize that the blood coagulation pathway and the role of Factor VII therein are well known, and therefore many 15 variants, both naturally occurring and engineered, as described above, are included in the present invention.

Methods for the expression and to determine the activity of Factor VII are well known in the art, and are described in, for example, U.S. Patent No. 4,784,950. Briefly, expression 15 of Factor VII, or variants thereof, can be accomplished in a variety of both prokaryotic and eukaryotic systems, including *E. coli*, CHO cells, BHK cells, insect cells using a baculovirus expression system, all of which are well known in the art.

Assays for the activity of a modified Factor VII prepared according to the methods of the present invention can be accomplished using methods well known in the art. As a non- 20 limiting example, Quick et al. (*Hemorrhagic Disease and Thrombosis*, 2nd ed., Leat Febiger, Philadelphia, 1966), describes a one-stage clotting assay useful for determining the biological activity of a Factor VII molecule prepared according to the methods of the present invention.

#### D. Factor IX

The present invention further encompasses a method for remodeling and/or modifying 25 Factor IX. As described above, Factor IX is vital in the blood coagulation cascade. A deficiency of Factor IX in the body characterizes a type of hemophilia (type B). Treatment of this disease is usually limited to intravenous transfusion of human plasma protein concentrates of Factor IX. However, in addition to the practical disadvantages of time and expense, 30 transfusion of blood concentrates involves the risk of transmission of viral hepatitis, acquired immune deficiency syndrome or thromboembolic diseases to the recipient.

While Factor IX has demonstrated itself as an important and useful compound for therapeutic applications, present methods for the production of Factor IX from recombinant cells (U.S. Patent No. 4,770,999) results in a product with a rather short biological life, an inaccurate glycosylation pattern that could potentially lead to immunogenicity, loss of function, an increased need for both larger and more frequent doses in order to achieve the same effect, and the like.

The nucleic and amino acid sequences of Factor IX is set forth herein as SEQ ID NO:9 and SEQ ID NO:10 (Figure 56A and 56B, respectively). The present invention is in no way limited to the sequences set forth herein. Factor IX variants are well known in the art, as described in, for example, U.S. Patent Nos. 4,770,999, 5,521,070 in which a tyrosine is replaced by an alanine in the first position, U.S. Patent No. 6,037,452, in which Factor XI is bound to an alkylene oxide group, and U.S. Patent No. 6,046,380, in which the DNA encoding Factor IX is modified in at least one splice site. As demonstrated herein, variants of Factor IX are well known in the art, and the present disclosure encompasses those variants known or to be developed or discovered in the future.

Methods for determining the activity of a modified Factor IX prepared according to the methods of the present invention can be carried out using the methods described above, or additionally, using methods well known in the art, such as a one stage activated partial thromboplastin time assay as described in, for example, Biggs (1972, Human Blood Coagulation Haemostasis and Thrombosis (Ed. 1), Oxford, Blackwell, Scientific, pg. 614). Briefly, to assay the biological activity of a Factor IX molecule developed according to the methods of the present invention, the assay can be performed with equal volumes of activated partial thromboplastin reagent, Factor IX deficient plasma isolated from a patient with hemophilia B using sterile phlebotomy techniques well known in the art, and normal pooled plasma as standard, or the sample. In this assay, one unit of activity is defined as that amount present in one milliliter of normal pooled plasma. Further, an assay for biological activity based on the ability of Factor IX to reduce the clotting time of plasma from Factor IX-deficient patients to normal can be performed as described in, for example, Proctor and Rapaport (1961, Amer. J. Clin. Path. 36: 212).

E. FSH

The present invention further includes a method for remodeling and/or modifying FSH. Human reproductive function is controlled in part by a family of heterodimeric human glycoprotein hormones which have a common 92 amino acid glycoprotein alpha subunit, but differ in their hormone-specific beta subunits. The family includes follicle-stimulating hormone (FSH), luteinizing hormone (LH), thyrotropin or thyroid-stimulating hormone (TSH), and human chorionic gonadotropin (hCG). Human FSH and LH are used therapeutically to regulate various aspects of metabolism pertinent to reproduction in the human female. For example, FSH partially purified from urine is used clinically to stimulate 5 follicular maturation in anovulatory women with anovulatory syndrome or luteal phase deficiency. Luteinizing hormone (LH) and FSH are used in combination to stimulate the development of ovarian follicles for *in vitro* fertilization. The role of FSH in the reproductive cycle is sufficiently well-known to permit therapeutic use, but difficulties have been 10 encountered due, in part, to the heterogeneity and impurity of the preparation from native 15 sources. This heterogeneity is due to variations in glycosylation pattern.

FSH is a valuable tool in both *in vitro* fertilization and stimulation of fertilization *in vivo*, but as stated above, its clinical efficacy has been hampered by inconsistency in glycosylation of the protein. It therefore seems apparent that a method for remodeling FSH will be of great benefit to the reproductive sciences.

20 FSH has been cloned and sequenced, the nucleic acid sequences of which are presented herein as SEQ ID NO:11, SEQ ID NO: 12, respectively (alpha subunit) and SEQ ID NO:13 and SEQ ID NO:14, respectively (beta subunit) (Figure 57A and 57B, respectively). The skilled artisan will readily appreciate that the present invention is not limited to the sequences depicted herein, as variants of FSH are well known in the art. As a 25 non-limiting example, U.S. Patent No. 5,639,640 describes the beta subunit comprising two different amino acid sequences and U.S. Patent No. 5,338,835 describes a beta subunit comprising an additional amino acid sequence of approximately twenty-seven amino acids derived from the beta subunit of human chorionic gonadotropin. Therefore, the present invention comprises FSH variants, both natural and engineered by the human hand, all well 30 known in the art.

Methods to express FSH in cells, both prokaryotic and eukaryotic, are well known in the art and abundantly described in the literature (U.S. Patent Nos. 4,840,896, 4,923,805, 5,156,957). Further, methods for evaluating the biological activity of a remodeled FSH molecule of the present invention are well known in the art, and are described in, for 5 example, U.S. Patent No. 4,589, 402, in which methods for determining the effect of FSH on fertility, egg production, and pregnancy rates is described in both non-human primates and human subjects.

#### F. EPO

10 The present invention further comprises a method of remodeling and/or modifying EPO. EPO is an acidic glycoprotein of approximately 34 kDa and may occur in three natural forms: alpha, beta, and asialo. The alpha and beta forms differ slightly in carbohydrate components but have the same potency, biological activity and molecular weight. The asialo form is an alpha or beta form with the terminal sialic acid removed. EPO is present in very 15 low concentrations in plasma when the body is in a healthy state wherein tissues receive sufficient oxygenation from the existing number of erythrocytes. This normal concentration is enough to stimulate replacement of red blood cells which are lost normally through aging. The amount of erythropoietin in the circulation is increased under conditions of hypoxia when oxygen transport by blood cells in the circulation is reduced. Hypoxia may be caused 20 by loss of large amounts of blood through hemorrhage, destruction of red blood cells by over-exposure to radiation, reduction in oxygen intake due to high altitudes or prolonged unconsciousness, or various forms of anemia. Therefore EPO is a useful compound for replenishing red blood cells after radiation therapy, anemia, and other life-threatening conditions.

25 In light of the importance of EPO in aiding in the recovery from a variety of diseases and disorders, the present invention is useful for the production of EPO with a natural, and therefore more effective saccharide component. EPO, as it is currently synthesized, lacks the full glycosylation complement, and must therefore be administered more frequently and in higher doses due to its short life in the body.

30 EPO has been cloned and sequenced, and the nucleotide and amino acid sequences are present herein as SEQ ID NO:15 and SEQ ID NO:16, respectively (Figure 58A and 58B,

respectively). It will be readily understood by one of skill in the art that the sequences set forth herein are only an example of the sequences encoding and comprising EPO. As an example, U.S. Patent No. 6,187,564 describes a fusion protein comprising the amino acid sequence of two or more EPO peptides, U.S. Patent Nos. 6,048,971 and 5,614,184 describe 5 mutant EPO molecules having amino acid substitutions at positions 101, 103, 104, and 108. U.S. Patent No. 5,106,954 describes a truncated EPO molecule, and U.S. Patent No. 5,888,772 describes an EPO analog with substitutions at position 33, 139, and 166. Therefore, the skilled artisan will realize that the present invention encompasses EPO and EPO derivatives and variants as are well documented in the literature and art as a whole.

10        Additionally, methods of expressing EPO in a cell are well known in the art. As exemplified in U.S. Patent Nos. 4,703,008, 5,688,679, and 6,376,218, among others, EPO can be expressed in prokaryotic and eukaryotic expression systems. Methods for assaying the biological activity of EPO are equally well known in the art. As an example, the Krystal assay (Krystal, 1983, Exp. Hematol. 11:649-660) can be employed to determine the activity 15 of EPO prepared according to the methods of the present invention. Briefly, the assay measures the effect of erythropoietin on intact mouse spleen cells. Mice are treated with phenylhydrazine to stimulate production of erythropoietin-responsive red blood cell progenitor cells. After treatment, the spleens are removed, intact spleen cells are isolated and incubated with various amounts of wild-type erythropoietin or the erythropoietin proteins described herein. After an overnight incubation, <sup>3</sup>H-thymidine is added and its incorporation 20 into cellular DNA is measured. The amount of <sup>3</sup>H-thymidine incorporation is indicative of erythropoietin-stimulated production of red blood cells via interaction of erythropoietin with its cellular receptor. The concentration of the erythropoietin protein of the present invention, as well as the concentration of wild-type erythropoietin, is quantified by competitive 25 radioimmunoassay methods well known in the art. Specific activities are calculated as international units measured in the Krystal assay divided by micrograms as measured as immunoprecipitable protein by radioimmunoassay.

#### G. GM-CSF

30        The present invention encompasses a method for the modification of GM-CSF. GM-CSF is well known in the art as a cytokine produced by activated T-cells, macrophages,

endothelial cells, and stromal fibroblasts. GM-CSF primarily acts on the bone marrow to increase the production of inflammatory leukocytes, and further functions as an endocrine hormone to initiate the replenishment of neutrophils consumed during inflammatory functions. Further GM-CSF is a macrophage-activating factor and promotes the 5 differentiation of Lagerhans cells into dendritic cells. Like G-CSF, GM-CSF also has clinical applications in bone marrow replacement following chemotherapy.

While G-CSF has demonstrated itself as an important and useful compound for therapeutic applications, present methods for the production of G-CSF from recombinant cells results in a product with a rather short biological life, an inaccurate glycosylation pattern 10 that could potentially lead to immunogenicity, loss of function, an increased need for both larger and more frequent doses in order to achieve the same effect, and the like.

GM-CSF has been isolated and cloned, the nucleic acid and amino acid sequences of which are presented as SEQ ID NO:17 and SEQ ID NO:18, respectively (Figure 59A and 15 59B, respectively). The present invention encompasses a method for modifying GM-CSF, particularly as it relates to the ability of GM-CSF to function as a potent and functional biological molecule. The skilled artisan, when equipped with the present disclosure and the teachings herein, will readily understand that the present invention provides compositions and methods for the modification of GM-CSF.

The present invention further encompasses GM-CSF variants, as well known in the 20 art. As an example, but in no way meant to be limiting to the present invention, a GM-CSF variant has been described in WO 86/06358, where the protein is modified for an alternative quaternary structure. Further, U.S. Patent No. 6,287,557 describes a GM-CSF nucleic acid sequence ligated into the genome of a herpesvirus for gene therapy applications. Additionally, European Patent Publication No. 0288809 (corresponding to PCT Patent 25 Publication No. WO 87/02060) reports a fusion protein comprising IL-2 and GM-CSF. The IL-2 sequence can be at either the N- or C-terminal end of the GM-CSF such that after acid cleavage of the fusion protein, GM-CSF having either N- or C-terminal sequence modifications can be generated. Therefore, GM-CSF derivatives, mutants, and variants are well known in the art, and are encompassed within the methods of the present invention.

30 The expression and activity of a modified GM-CSF molecule of the present invention can be assayed using methods well known in the art, and as described in, for example, U.S.

Patent No. 4,810,643. As an example, activity can be measured using radio-labeled thymidine uptake assays. Briefly, human bone marrow from healthy donors is subjected to a density cut with Ficoll-Hypaque (1.077 g/ml, Pharmacia, Piscataway, NJ) and low density cells are suspended in Iscove's medium (GIBCO, La Jolla, CA) containing 10% fetal bovine serum, glutamine and antibiotics. About  $2 \times 10^4$  human bone marrow cells are incubated with either control medium or the GM-CSF or the present invention in 96-well flat bottom plates at about 37° C in 5% CO<sub>2</sub> in air for about 2 days. Cultures are then pulsed for about 4 hours with 0.5 μCi/well of <sup>3</sup>H-thymidine (New England Nuclear, Boston, Mass.) and uptake is measured as described in, for example, Ventua, et al.(1983, Blood 61:781). An increase in <sup>3</sup>H-thymidine incorporation into human bone marrow cells as compared to bone marrow cells treated with a control compound is an indication of a active and viable GM-CSF compound.

#### H. IFN-gamma

It is an object of the present invention to encompass a method of modifying and/or remodeling IFN-gamma. IFN-gamma, otherwise known as Type II interferon, in contrast to IFN alpha and IFN beta, is a homodimeric glycoprotein comprising two subunits of about 21-24 kDa. The size variation is due to variable glycosylation patterns, usually not replicated when reproduced recombinantly in various expression systems known in the art. IFN-gamma is a potent activator of macrophages, increases MHC class I molecule expression, and to a lesser extent, a MHC class II molecule stimulatory agent. Further, IFN-gamma promotes T-cell differentiation and isotype switching in B-cells. IFN-gamma is also well documented as a stimulator of neutrophils, NK cells, and antibody responses leading to phagocyte-mediated clearance. IFN-gamma has been proposed as a treatment to be used in conjunction with infection by intracellular pathogens, such as tuberculosis and leishmania, and also as an anti-proliferative therapeutic, useful in conditions with abnormal cell proliferation as a hallmark, such as various cancers and other neoplasias.

IFN-gamma has demonstrated potent immunological activity, but due to variations in glycosylation from systems currently used to express IFN-gamma, the potency, efficacy, biological half-life, and other important factors of a therapeutic have been variable at best. The present invention encompasses methods to correct this crucial defect.

The nucleotide and amino acid sequences of IFN-gamma are presented herein as SEQ ID NO:19 and SEQ ID NO:20, respectively (Figure 60A and 60B, respectively). It will be readily understood that the sequences set forth herein are in no way limiting to the present invention. In contrast, variants, derivatives, and mutants of IFN-gamma are well known to the skilled artisan. As an example, U.S. Patent No. 6,083,724 describes a recombinant avian IFN-gamma and U.S. Patent No. 5,770,191 describes C-terminus variants of human IFN-gamma. In addition, U.S. Patent No. 4,758,656 describes novel IFN-gamma derivatives, and methods of synthesizing them in various expression systems. Therefore, the present invention is not limited to the sequences of IFN-gamma disclosed elsewhere herein, but 10 encompasses all derivatives, variants, muteins, and the like well known in the art.

Expression systems for IFN-gamma are equally well known in the art, and include prokaryotic and eukaryotic systems, as well as plant and insect cell preparations, methods of which are known to the skilled artisan. As an example, U.S. Patent No. 4,758,656 describes a system for expressing IFN-gamma derivatives in *E. coli*, whereas U.S. Patent No. 4,889,803 15 describes an expression system employing Chinese hamster ovary cells and an SV40 promoter.

Assays for the biological activity of a remodeled IFN-gamma prepared according to the methods disclosed herein will be well known to one of skill in the art. Biological assays for IFN-gamma expression can be found in, for example, U.S. Patent No. 5,807,744. Briefly, 20 IFN-gamma is added to cultures of CD34<sup>++</sup>CD38<sup>-</sup> cells (100 cells per well) stimulated by cytokine combinations to induce proliferation of CD34<sup>++</sup>CD38<sup>-</sup> cells, such as IL-3, c-kit ligand and either IL-1, IL-6 or G-CSF. Cell proliferation, and generation of secondary colony forming cells will be profoundly inhibited in a dose dependent way, with near complete inhibition occurring at 5000 U/milliliter of IFN-gamma. As a confirmatory test to 25 the inhibitory effect of IFN-gamma, addition of IFN-gamma antibodies can be performed as a control.

#### I. alpha-Protease inhibitor ( $\alpha$ -antitrypsin)

The present invention further includes a method for the remodeling of alpha-protease 30 inhibitor (A-1-PI,  $\alpha$ -1-antitrypsin or  $\alpha$ -1-trypsin inhibitor), also known as alpha-antitrypsin. A-1-PI is a glycoprotein having molecular weight of 53 kDa. A-1-PI plays a role in

controlling tissue destruction by endogenous serine proteases, and is the most pronounced serine protease inhibitor in blood plasma. In particular, A-1-PI inhibits various elastases including neutrophil elastase. Elastase is a protease which breaks down tissues, and can be particularly problematic when its activity is unregulated in lung tissue. This protease  
5 functions by breaking down foreign proteins. However, when API is not present in sufficient quantities to regulate elastase activity, the elastase breaks down lung tissue. In time, this imbalance results in chronic lung tissue damage and emphysema. In fact, a genetic deficiency of A-1-PI has been shown to be associated with premature development of pulmonary emphysema. A-1-PI replenishment has been successfully used for treatment of  
10 this form of emphysema. Further, a deficiency of A-1-PI may also contribute to the aggravation of other diseases such as cystic fibrosis and arthritis, where leukocytes move in to the lungs or joints to fight infection.

Therefore, A-1-PI could conceivably be used to treat diseases where an imbalance between inhibitor and protease(s), especially neutrophil elastase, is causing progression of a  
15 disease state. Antiviral activity has also been attributed to A-1-PI. In light of this, it logically follows that the present invention is useful for the production of A-1-PI that is safe, effective, and potent in the ever changing atmosphere of the lungs.

A-1-PI has been cloned and sequenced, and is set forth in SEQ ID NO:21 and SEQ ID NO:22 (Figure 61A and 61B, respectively). As is understood by one of skill in the art,  
20 natural and engineered variants of A-1-PI exist, and are encompassed in the present invention. As an example, U.S. Patent No. 5,723,316 describes A-1-PI derivatives having amino acid substitutions at positions 356-361 and further comprises an N-terminal extension of approximately three amino acids. U. S. Patent No. 5,674,708 describes A-1-PI analogs with amino acid substitutions at position 358 in the primary amino acid sequence. The  
25 skilled artisan will readily realize that the present invention encompasses A-1-PI variants, derivatives, and mutants known or to be discovered.

Methods for the expression and determination of activity of a remodeled A-1-PI produced according to the methods of the present invention are well known in the art, and are described in, for example, U.S. Patent No. 5,674,708 and U.S. Patent No. 5,723,316. Briefly,  
30 biological activity can be determined using assays for antichymotrypsin activity by measuring the inhibition of the chymotrypsin-catalyzed hydrolysis of substrate N-suc-Ala--

Ala-Pro-Phe-p-nitroanilide (0.1 ml of a 10 mM solution in 90% DMSO), as described in, for example, DelMar et al. (1979, Anal. Biochem. 99: 316). A typical chymotrypsin assay contains, in 1.0 milliliters: 100 mM Tris-Cl buffer, pH 8.3, 0.005% (v/v) Triton X-100, bovine pancreatic chymotrypsin (18 kmmol) and A-1-PI of the present invention. The assay mixture is pre-incubated at room temperature for 5 minutes, substrate (0.01 ml of a 10 mM solution in 90% DMSO) is added and remaining chymotrypsin activity is determined by the rate of change in absorbance at 410nm caused by the release of p-nitroaniline. Measurements of optical absorbance are conducted at 25° C using a spectrophotometer fitted with a temperature controlled sample compartment.

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#### J. Glucocerebrosidase

The invention described herein further includes a method for the modification of glucocerebrosidase. Glucocerebrosidase is a lysosomal glycoprotein enzyme which catalyzes the hydrolysis of the glycolipid glucocerebroside to glucose and ceramide. Variants of glucocerebrosidase are sold commercially as Cerezyme™ and Ceredase™, and is an approved therapeutic for the treatment of Gaucher's disease. Ceredase™ is a placental derived form of glucocerebrosidase with complete N-linked structures. Cerezyme™ is a recombinant variant of glucocerebrosidase which is 497 amino acids in length and is expressed in CHO cells. The 4 N-linked glycans of Cerezyme have been modified to terminate in the trimannose core.

20 Glucocerebrosidase is presently produced in recombinant mammalian cell cultures, and therefore reflects the glycosylation patterns of those cells, usually rodent cells such as Chinese hamster ovary cells or baby hamster kidney cells, which differ drastically from those of human glycosylation patterns, leading to, among other things, immunogenicity and lack of potency.

25 The nucleic acid and amino acid sequences of glucocerebrosidase are set forth herein as SEQ ID NO: 23 and 24 (Figure 62A and 62B, respectively). However, as will be appreciated by the skilled artisan, the sequences represented herein are prototypical sequences, and do not limit the invention. In fact, variants of glucocerebrosidase are well known, and are described in, for example, U.S. Patent 6,015,703 describes enhanced production of glucocerebrosidase analogs and variants thereof. Further, U.S. Patent No.

6,087,131 describes the cloning and sequencing of yet another glucocerebrosidase variant. It is the intention of the present invention to encompass these and other derivatives, variants, and mutants known or to be discovered in the future.

Methods for the expression of glucocerebrosidase are well known in the art using

- 5 standard techniques, and are described in detail in, for example, U.S. Patent No. 6,015,703. Assays for the biological efficacy of a glucocerebrosidase molecule prepared according to the methods of the present invention are similarly well known in the art, and a mouse Gaucher's disease model for evaluation and use of a glucocerebrosidase therapeutic is described in, for example, Marshall et al. (2002, Mol. Ther. 6:179).

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#### K. TPA

- The present invention further encompasses a method for the remodeling of tissue-type activator (TPA). TPA activates plasminogen to form plasmin which dissolves fibrin, the main component of the protein substrate of the thrombus. TPA preparations were developed  
15 as a thrombolytic agents having a very high selectivity toward the thrombus in the thrombolytic treatment for thrombosis which causes myocardial infarction and cerebral infarction.

- Further, various modified TPA's have been produced by genetic engineering for the purpose of obtaining higher affinity to fibrin and longer half-life in blood than that of natural  
20 TPA. The modified TPA's produced from prokaryotes are not glycosylated unlike the natural TPA. TPA's are proteins that are generally extremely difficult to solubilize in water. In particular, the modified TPA's are more difficult to solubilize in water than natural TPA, making very difficult the preparation of modified TPA's. Modified TPA's are thus difficult to dissolve in water at the time of the administration to a patient. However, the modified TPA's  
25 have various advantages, such as increased affinity for fibrin and longer half-life in blood. It is the object of the present invention to increase the solubility of modified TPA's.

- The nucleic and amino acid sequences of TPA are set forth herein as SEQ ID NO:25 and SEQ ID NO:26, respectively (Figure 63A and 63B, respectively). As described above, variants of TPA have been constructed and used in therapeutic applications. For example,  
30 U.S. Patent 5,770,425 described TPA variants in which some of all of the fibrin domain has been deleted. Further, U.S. Patent 5,736,134 describes TPA in which modifications to the

amino acid at position 276 are disclosed. The skilled artisan, when equipped with the present disclosure and the teachings herein, will readily realize that the present invention comprises the TPA sequences set forth herein, as well as those variants well known to one versed in the literature.

- 5       The expression of TPA from a nucleic acid sequence encoding the same is well known in the art, and is described, in detail, in, for example, U.S. Patent No. 5,753,486. Assays for determining the biological properties of a TPA molecule prepared according to the methods of the present invention are similarly well known in the art. Briefly, a TPA molecule synthesized as disclosed elsewhere herein can be assayed for their ability to lyse  
10 fibrin in the presence of saturating concentrations of plasminogen, according to the method of Carlsen et al. (1988, Anal. Biochem. 168: 428). The *in vitro* clot lysis assay measures the activity of tissue-type activators by turbidimetry using a microcentrifugal analyzer. A mixture of thrombin and TPA is centrifuged into a mixture of fibrinogen and plasminogen to initiate clot formation and subsequent clot dissolution. The resultant profile of absorbance  
15 versus time is analyzed to determine the assay endpoint. Activities of the TPA variants are compared to a standard curve of TPA. The buffer used throughout the assay is 0.06M sodium phosphate, pH 7.4 containing 0.01% (v/v) TWEEN 80 and 0.01% (w/v) sodium azide. Human thrombin is at a concentration of about 33 units/ml. Fibrinogen (at 2.0 mg/ml clottable protein) is chilled on wet ice to precipitate fibronectin and then gravity filtered.  
20 Glu-plasminogen is at a concentration of 1 mg/ml. The analyzer chamber temperature is set at 37° C. The loader is set to dispense 20 microliters of TPA (about 500 nanograms/milliliter to about 1.5 micrograms per milliliter) as the sample for the standard curve, or 20 microliters of variant TPAs at a concentration to cause lysis within the range of the standard curve. Twenty microliters of thrombin as the secondary reagent, and 200 microliters of a 50:1 (v/v)  
25 fibrinogen: plasminogen mixture as the primary reagent. The absorbance/time program is used with a 5 min incubation time, 340-nanometer-filter and 90 second interval readings.

L. IL-2

The present invention further encompasses a method for the remodeling and modification of IL-2. IL-2 is the main growth factor of T lymphocytes and increases the humoral and cellular immune responses by stimulating cytotoxic CD8 T cells and NK cells.

IL-2 is therefore crucial in the defense mechanisms against tumors and viral infections. IL-2 is also used in therapy against metastatic melanoma and renal adenocarcinoma, and has been used in clinical trials in many forms of cancer. Further, IL-2 has also been used in HIV infected patients where it leads to a significant increase in CD4 counts.

Given the success IL-2 has demonstrated in the management and treatment of life-threatening diseases such as various cancers and AIDS, it follows that the methods of the present invention would be useful for developing an IL-2 molecule that has a longer biological half-life, increased potency, and in general, a therapeutic profile more similar to wild-type IL-2 as it is synthesized secreted in the healthy human.

IL-2 has been cloned and sequenced, and the nucleic acid and amino acid sequences are presented herein as SEQ ID NO:27 and SEQ ID NO:28 (Figure 64A and 64B, respectively). The present invention should in no way be construed as limited to the IL-2 nucleic acid and amino acid sequences set forth herein. Variants of IL-2 are described in, for example, U.S. Patent No. 6,348,193, in which the asparagine at position 88 is substituted for arginine, and in U.S. Patent No. 5,206,344, in which a polymer comprising IL-2 variants with various amino acid substitutions is described. The present invention encompasses these IL-2 variants and others well known in the art.

Methods for the expression and to determine the activity of IL-2 are well known in the art, and are described in, for example, U.S. Patent No. 5,417,970. Briefly, expression of IL-2, or variants thereof, can be accomplished in a variety of both prokaryotic and eukaryotic systems, including *E. coli*, CHO cells, BHK cells, insect cells using a baculovirus expression system, all of which are well known in the art.

Assays for the activity of a modified IL-2 prepared according to the methods of the present invention can proceed as follows. Peripheral blood lymphocytes can be separated from the erythrocytes and granulocytes by centrifuging on a Ficoll-Hypaque (Pharmacia, Piscataway, NJ) gradient by the method described in, for example, A. Boyum et al. (*Methods in Enzymology*, 1984, Vol. 108, page 88, Academic Press, Inc.). Lymphocytes are subsequently washed about three times in culture medium consisted of RPMI 1640 (Gibco-BRL, La Jolla, CA) plus 10% AB human serum (CTS Purpan, Toulouse, France) inactivated by heat (1 hour at 56° C), 2 mM sodium pyruvate, 5 mM HEPES, 4 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin B (complete medium).

Adhesive cells (monocytes and macrophages) are eliminated by adhesion to plastic and the remainder of the cells are suspended in complete medium at a concentration of about 5 to 10 X10<sup>5</sup> cells per milliliter and seeded in culture flasks at a density of about 1-2 X 10<sup>5</sup> cells per square centimeter. Flasks are then incubated at 37° C in a 5% CO<sub>2</sub> atmosphere for about 1 hour, after which the non-adhesive lymphocytes are recovered by aspiration after gentle agitation of the culture flasks.

Non-adhesive lymphocytes are washed once and cultivated at a concentration of about 10<sup>5</sup> cells per milliliter in complete medium in the presence of the IL-2 of the present invention for about 48 hours in an incubator as described above. The cells are then washed once.

The cytotoxic activity of the cells is evaluated after about 4 hours of contact with target cells of the human T lymphoid line C8166-45/C63 (HT1 cells) resistant to NK cell cytotoxicity, as described by Salahuddin et al. (1983, Virology 129: 51-64; 1984, Science: 223, 703-707). 6 X 10<sup>5</sup> HT1 cells are radio-tagged with about 200 µCi of <sup>51</sup>Cr (sodium chromate, Amersham, Arlington Heights, IL) at 37° C for about 1 hour in complete medium without serum, and then washed several times. The target cells and effective cells are distributed in round-bottomed microfiltration plates with varying ratios of effective cells to target cells (50:1, 10:1, 1:1). The microfiltration plates are centrifuged and, after incubation as described above, the supernatant from each well is recovered and the radioactivity is measured using a gamma counter. Cytotoxicity is determined from the quantity of <sup>51</sup>Cr released by dead target cells. Non-specific cytotoxicity is determined from the amount of radioactivity spontaneously released from the target cells in the absence of effective cells.

The present method is just one of many well known in the art for measuring the cytotoxicity of effector cells, and is should not be construed as limiting to the present invention.

#### M. Factor VIII

The invention further encompasses a method for the remodeling and modification of Factor VIII. As described earlier for Factor VII and Factor IX, Factor VIII is a critical component of the blood coagulation pathway. Human Factor VIII, (antihemophilic factor; FVIII:C) is a human plasma protein consisting of 2 peptides (light chain molecular weight of 80 kDa and

heavy chain molecular weight variable from 90 to 220 kDa, depending on glycosylation state). It is an essential cofactor in the coagulation pathway and is required for the conversion of Factor X into its active form (Factor Xa). Factor VIII circulates in plasma as a non-covalent complex with von Willibrand Factor (aka FVIII:RP), a dimer of a 2050 aa peptide (See, U.S. Patent No. 6,307,032). Blood concentrations of Factor VIII below 20% of normal cause a bleeding disorder designated hemophilia A. Factor VIII blood levels less than 1% result in a severe bleeding disorder, with spontaneous joint bleeding being the most common symptom.

Similar to other blood coagulation factors, Factor VIII is a therapeutic with a great deal of potential for the treatment of various bleeding disorders, such as hemophilia A and hemophilia B. Due to the glycosylation of the heavy chain, current methods for the preparation of Factor VIII from recombinant cells results in a product that is not as effective as natural Factor VIII. Purification methods from human plasma result in a crude composition that is less effective and more difficult to prepare than recombinant Factor VIII.

The current invention seeks to improve this situation.

The nucleic acid and amino acid sequences of Factor VIII are presented herein as SEQ ID NO:29 and SEQ ID NO:30, respectively (Figure 65A and 65B, respectively). The art is rife with variants of Factor VIII, as described in, for example, U.S. Patent No. 5,668,108, in which the aspartic acid at position 1241 is replaced by a glutamic acid with the accompanying nucleic acid changes as well. U.S. Patent No. 5,149,637 describes a Factor VIII variants comprising the C-terminal fraction, either glycosylated or unglycosylated, and U.S. Patent No. 5,661,008 describes a Factor VIII variant comprising amino acids 1-740 linked to amino acids 1649 to 2332 by at least 3 amino acid residues. Therefore, variants, derivatives, modifications and complexes of Factor VIII are well known in the art, and are encompassed in the present invention.

Expression systems for the production of Factor VIII are well known in the art, and include prokaryotic and eukaryotic cells, as exemplified in U.S. Patent Nos. 5,633,150, 5,804,420, and 5,422,250.

To determine the biological activity of a Factor VIII molecule synthesized according to the methods of the present invention, the skilled artisan will recognize that the assays described herein for the evaluation of Factor VII and Factor IX are applicable to Factor VIII.

N. Urokinase

The present invention also includes a method for the remodeling and/or modification of urokinase. Urokinase is a serine protease which activates plasminogen to plasmin. The 5 protein is synthesized in a variety of tissues including endothelium and kidney, and is excreted in trace amounts into urine. Purified urokinase exists in two active forms, a high molecular weight form (HUK; approximately 50 kDa) and a low molecular weight form (LUK; approximately 30 kDa). LUK has been shown to be derived from HUK by a proteolysis after lysine 135, releasing the first 135 amino acids from HUK. Conventional 10 wisdom has held that HUK or LUK must be converted to proteolytically active forms by the proteolytic hydrolysis of a single chain precursor, also termed prourokinase, between lysine 158 and isoleucine 159 to generate a two-chain activated form (which continues to correspond to either HUK or LUK). The proteolytically active urokinase species resulting 15 from this hydrolytic clip contains two amino acid chains held together by a single disulfide bond. The two chains formed by the activation clip are termed the A or A<sub>1</sub> chains (HUK or LUK, respectively), and the B chain comprising the protease domain of the molecule.

Urokinase has been shown to be an effective thrombolytic agent. However, since it is produced naturally in trace quantities the cost of the enzyme is high for an effective dosage. Urokinase has been produced in recombinant cell culture, and DNA encoding urokinase is 20 known together with suitable vectors and host microorganisms. Present compositions comprising urokinase and methods for producing urokinase recombinantly are hampered by a product that has deficient glycosylation patterns, and given the complex proteolytic cleavage events surrounding the activation of urokinase, this aberrant glycosylation leads to a less effective and less potent product.

25 The sequence of the nucleotides encoding the primary amino acid chain of urokinase are depicted in SEQ ID NO:33 and SEQ ID NO:34 (Figure 66A and 66B, respectively). Variants of urokinase are well known in the art, and therefore the present invention is not limited to the sequences set forth herein. In fact, the skilled artisan will readily realize that urokinase variants described in, for example U.S. Patent Nos. 5,219,569, 5,648,253, and 30 4,892,826, exist as functional moieties, and are therefore encompassed in the present invention.

The expression and evaluation of a urokinase molecule prepared according to the methods of the present invention are similarly well known in the art. As a non-limiting example, the expression of urokinase in various systems is detailed in U.S. Patent No. 5,219,569. An assay for determining the activity and functionality of a urokinase prepared in accordance to the methods set forth herein are described throughout the literature, and are similar to assays for other plasminogen and fibrin related assays described elsewhere throughout. One example of an assay to determine the activity of an urokinase molecule synthesized as described herein can be as described in, for example, Ploug, et al. (1957, Biochim. Biophys. Acta 24: 278-282), using fibrin plates comprising 1.25% agarose, 4.1 mg/ml human fibrinogen, 0.3 units/ml of thrombin and 0.5 µg/ml of soybean trypsin inhibitor.

#### O. Human DNase

The present invention further encompasses a method for the remodeling and/or modification of recombinant human DNase. Human DNase I has been tested as a therapeutic agent and was shown to diminish the viscosity of cystic fibrosis mucus *in vitro*. It has been determined that purulent mucus contains about 10-13 mg/ml of DNA, an ionic polymer predicted to affect the rheologic properties of airway fluids. Accordingly, bovine pancreatic DNase I, an enzyme that degrades DNA, was tested as a mucolytic agent many years ago but did not enter clinical practice, because of side effects induced by antigenicity and/or contaminating proteases. Recombinant human DNase is currently used as a therapeutic agent to alleviate the symptoms of diseases such as cystic fibrosis.

Similar to DNase derived from bovine sources, recombinant human DNase poses some problems, mostly due to lowered efficacy due to improper glycosylation imparted by mammalian expression systems currently in use. The present invention describes a method for remodeling DNase, leading to increased efficacy and better therapeutic results.

The nucleotide and amino acid sequences of human DNase are presented herein as SEQ ID NO:39 and SEQ ID NO:40 (Figure 67A and 67B, respectively). Variants of the peptide comprising DNase are well known in the art. As an example, U.S. Patent No. 6,348,343 describes a human DNase with multiple amino acid substitutions throughout the primary structure. Additionally, U.S. Patent No. 6,391,607 describes a hyperactive variant of

DNase with multiple amino acid substitutions at positions 9, 14, 74, 75, and 205. The present examples, and others well known in the art or to be discovered in the future are encompassed in the present invention.

Expression systems for producing a DNase peptide are well known to the skilled

- 5 artisan, and have been described in prokaryotic and eukaryotic systems. For example, PCT Patent Publication No. WO 90/07572 describes these methods in considerable detail.

Assays to determine the biological activity of a DNase molecule developed according to the methods of the present invention are well known in the art. As an example, but in no way meant to be limiting to the present invention, an assay to determine the DNA-hydrolytic

- 10 activity of human DNase I is presented herein. Briefly, two different plasmid digestion assays are used. The first assay ("supercoiled DNA digestion assay") measures the conversion of supercoiled double-stranded plasmid DNA to relaxed (nicked), linear, and degraded forms. The second assay ("linear DNA digestion assay") measured the conversion of linear double-stranded plasmid DNA to degraded forms. Specifically, DNase prepared according to the methods of the present invention is added to 160 microliters of a solution comprising 25 micrograms per milliliter of either supercoiled plasmid DNA or EcoRI-digested linearized plasmid DNA in 25 mM HEPES, pH 7.1, 100 µg/ml bovine serum albumin, 1 mM MgCl<sub>2</sub>, 2.5 mM CaCl<sub>2</sub>, 150 mM NaCl, and the samples are incubated at room temperature. At various times, aliquots of the reaction mixtures are removed and quenched by the addition of 25 mM EDTA, together with xylene cyanol, bromophenol blue, and glycerol. The integrity of the plasmid DNA in the quenched samples is analyzed by electrophoresis of the samples on agarose gels. After electrophoresis, the gels are stained with a solution of ethidium bromide and the DNA in the gels is visualized by ultraviolet light. The relative amounts of supercoiled, relaxed, and linear forms of plasmid DNA are determined by scanning the gels with a fluorescent imager (such as the Molecular Dynamics Model 575 FluorImager) and quantitating the amount of DNA in the bands of the gel that correspond to the different forms.

#### P. Insulin

- 30 The invention further includes a method for remodeling insulin. Insulin is well known as the most effective treatment for type I diabetes, in which the beta islet cells of the

pancreas do not produce insulin for the regulation of blood glucose levels. The ramifications of diabetes and uncontrolled blood glucose include circulatory and foot problems, and blindness, not to mention a variety of other complications that either result from or are exacerbated by diabetes.

5 Prior to the cloning and sequencing of human insulin, porcine insulin was used as a treatment for diabetes. Insulin is now produced recombinantly, but the short, 51 amino acid sequence of the mature molecule is a complex structure comprising multiple sulfide bonds. Current methods to recombinantly produce insulin result in a product that lacks similarity to the native protein as produced in healthy non-diabetic subjects. The present invention seeks  
10 to repair this flaw.

The nucleotide and amino acid sequence of human insulin is portrayed in SEQ ID NO:43 and SEQ ID NO:44, respectively (Figure 68A and 68B, respectively). Variants of insulin are abundant throughout the art. U.S. Patent No. 6,337,194 describes insulin fusion protein analogs, U.S. Patent No. 6,323,311 describes insulin derivatives comprising a cyclic  
15 anhydride of a dicarboxylic acid, and U.S. Patent No. 6,251,856 describes an insulin derivative comprising multiple amino acid substitutions and a lipophilic group. The skilled artisan will recognize that the following examples of insulin derivatives are in no way exhaustive, but simply represent a small sample of those well known in the art. Therefore, the present invention comprises insulin derivatives known or to be discovered.

20 Expression systems for the production of insulin are well known in the art, and can be accomplished using molecular biology techniques as described in, for example, Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York).

Assays to determine the functionality of an insulin molecule prepared according to the  
25 methods of the present invention are similarly well known in the art. For example, an *in vivo* model of glucose depression can be used to evaluate the biological activity of insulin synthesized using the methods of the present invention. Useful for this purpose is a rat model. The animals are fasted overnight (16 hours) prior to the experiment, and then anesthetized with intraperitoneally administered sodium pentobarbital or another suitable  
30 anesthetic such as ketamine. Each animal receives an i.v. injection (tail vein) of the particular insulin derivative (20 µg/ml/kg). Blood samples are taken from the jugular vein 15 and 5

minutes before injection and 15, 30, 60, 90, 120, 180, and 240 minutes after injection. Blood glucose levels are measured with a blood glucose monitor, available from a variety of commercial suppliers.

5       O. Hepatitis B Vaccines (HBsAg)

The present invention further comprises a method for the remodeling the antigen used in hepatitis B vaccines (HBsAg or Hepatitis B sAg). HBsAg is a recombinantly produced surface antigen of the hepatitis B S-protein, and is used to illicit an immune response to the hepatitis B virus, an increasing dangerous virus that results in, among other things, liver disease including cirrhosis and carcinoma, and results in over 1 million deaths worldwide annually. Currently the HBsAg vaccine is administered three times over a six month interval to illicit a protective and neutralizing immune response.

10      HBsAg is currently produced in yeast strains, and therefore reflects the glycosylation patterns native to a fungus. The present invention provides a method to remodel HBsAg, resulting in among other things, improved immunogenicity, antibodies with improved affinity 15 for the virus, and the like.

15      The sequences of the S-protein from a Hepatitis B virus (HBsAg) nucleic acid and primary amino acid chain are set forth herein as SEQ ID NO:45 and SEQ ID NO:46 (Figure 69A and 69B, respectively). The nucleotide is 1203 bases in length. The amino acid is 400 residues long. The last 226 amino acid residues are the small S-antigen, which is used in the 20 GlaxoSmithKline vaccine and the Merck vaccine. Fifty-five amino acids upstream from the small S-antigen is the Pre-S start codon. The Pre-S + S regions are the middle S antigen, which is used in the Aventis Pasteur vaccine. From the first start codon to the Pre-S start codon comprises the rest of the S-protein, and is called the large S-protein. This is but one 25 example of the HBsAg used in vaccines, and other subtypes are well known, as exemplified in GenBank Acc Nos.: AF415222, AF415221, AF415220, and AF415219. The sequences presented herein are simply examples of HBsAg known in the art. Similar antigens have been isolated from other strains of hepatitis B virus, and may or may not have been evaluated for antigenicity and potential as vaccine candidates. The present invention therefore 30 encompasses hepatitis B vaccine S-protein surface antigens known or to be discovered.

Expression of an HBsAg in an expression system is a routine procedure for one of skill in the art, and is described in, for example, U.S. Patent No. 5,851,823. Assays for the immunogenicity of a vaccine are well known in the art, and comprise various tests for the production of neutralizing antibodies, and employ techniques such as ELISA, neutralization assays, Western blots, immunoprecipitation, and the like. Briefly, a sandwich ELISA for the detection of effective anti-HBsAg antibodies is described. The Enzygnost HBsAg assay (Aventis Behring, King of Prussia, PA) is used for such methods. Wells are coated with anti-HBs. Serum plasma or purified protein and appropriate controls are added to the wells and incubated. After washing, peroxidase-labeled antibodies to HBsAg are reacted with the remaining antigenic determinants. The unbound enzyme-linked antibodies are removed by washing and the enzyme activity on the solid phase is determined by methods well known in the art. The enzymatically catalyzed reaction of hydrogen peroxide and chromogen is stopped by adding diluted sulfuric acid. The color intensity is proportional to the HBsAg concentration of the sample and is obtained by photometric comparison of the color intensity of the unknown samples with the color intensities of the accompanying negative and positive control sera.

#### R. Human Growth Hormone

The present invention further encompasses a method for the remodeling of human growth hormone (HGH). The isoform of HGH which is secreted in the human pituitary, consists of 191 amino acids and has a molecular weight of about 21,500. The isoform of HGH which is made in the placenta is a glycosylated form. HGH participates in much of the regulation of normal human growth and development, including linear growth (somatogenesis), lactation, activation of macrophages, and insulin-like and diabetogenic effects, among others.

HGH is a complex hormone, and its effects are varied as a result of interactions with various cellular receptors. While compositions comprising HGH have been used in the clinical setting, especially to treat dwarfism, the efficacy is limited by the glycosylation structure of HGH produced recombinantly.

The nucleic and amino acid sequence of HGH are set forth elsewhere herein as SEQ ID NO:47 and SEQ ID NO:48 (Figure 70A and 70B, respectively). The skilled artisan will

recognize that variants, derivatives, and mutants of HGH are well known. Examples can be found in U.S. Patent No. 6,143,523 where amino acid residues at positions 10, 14, 18, 21, 167, 171, 174, 176 and 179 are substituted, and in U.S. Patent No. 5,962,411 describes splice variants of HGH. The present invention encompasses these HGH variants known in the art of  
5 to be discovered.

Methods for the expression of HGH in recombinant cells is described in, for example, U.S. Patent No. 5,795,745. Methods for expression of HGH in, *inter alia*, prokaryotes, eukaryotes, insect cell systems, plants, and *in vitro* translation systems are well known in the art.

10 An HGH molecule produced using the methods of the current invention can be assayed for activity using a variety of methods known to the skilled artisan. For example, U.S. Patent 5,734,024 describes a method to determine the biological functionality of an expressed HGH.

15 S. Antibodies

The present invention further comprises a method for the remodeling of various chimeric antibody preparations, including, chimeric TNFR, chimeric anti-glycoprotein IIb/IIIa, chimeric anti-HER2, chimeric anti-RSV, chimeric anti-CD20, and chimeric anti-TNF. Chimeric antibody preparations comprise a human Fc portion from an IgG antibody  
20 and the variable regions from a monoclonal antibody specific for an antigen. Other preparations comprise a receptor, for example the 75 kDa TNF receptor, fused to a human IgG Fc portion. These molecules further include Fab fragments comprising light and heavy chains from human and mice. A chimeric TNFR is useful in the treatment of inflammatory diseases, such as rheumatoid arthritis. Chimeric anti-glycoprotein IIb/IIIa is useful in the  
25 treatment of cardiac abnormalities, blood clotting, and platelet function disturbances. A chimeric anti-HER2 is useful as a treatment for breast cancer, chimeric anti-RSV is useful for the treatment of respiratory syncytial virus, chimeric anti-CD20 is useful for the treatment of Non-Hodgkin's lymphoma, and chimeric anti-TNF is used for treatment of Crohn's disease.

While these chimeric antibodies have proved useful in the management of varied  
30 diseases, administration has to be fairly frequent and at fairly high doses due to the relatively short half-life of a recombinant protein produced in rodent cells. While a majority of the

chimeric antibody is human, and therefore regarded as "self" by the immune system, they are degraded and destroyed due to non-native glycosylation patterns. The present invention proposes to repair this problem, greatly increasing the efficacy of these novel medicines.

Antibodies and Methods of their Generation

5        The term "antibody," as used herein, refers to an immunoglobulin molecule which is able to specifically bind to a specific epitope on an antigen. Antibodies can be intact immunoglobulins derived from natural sources or from recombinant sources and can be immunoreactive portions of intact immunoglobulins. Antibodies are typically tetramers of immunoglobulin molecules. The antibodies in the present invention may exist in a variety of forms including, for example, polyclonal antibodies, monoclonal antibodies, Fv, Fab and F(ab)<sub>2</sub>, as well as single chain antibodies and humanized antibodies (Harlow et al., 1999, Using Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory Press, NY; Harlow et al., 1989, Antibodies: A Laboratory Manual, Cold Spring Harbor, New York; Houston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; Bird et al., 1988, Science 242:423-426).

15       By the term "synthetic antibody" as used herein, is meant an antibody which is generated using recombinant DNA technology, such as, for example, an antibody expressed by a bacteriophage as described herein. The term should also be construed to mean an antibody which has been generated by the synthesis of a DNA molecule encoding the antibody and which DNA molecule expresses an antibody peptide, or an amino acid sequence specifying the antibody, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

Monoclonal antibodies directed against full length or peptide fragments of a peptide or peptide may be prepared using any well known monoclonal antibody preparation procedures, such as those described, for example, in Harlow et al. (1988, In: Antibodies, A Laboratory Manual, Cold Spring Harbor, NY) and in Tuszyński et al. (1988, Blood, 72:109-115). Quantities of the desired peptide may also be synthesized using chemical synthesis technology. Alternatively, DNA encoding the desired peptide may be cloned and expressed from an appropriate promoter sequence in cells suitable for the generation of large quantities of peptide. Monoclonal antibodies directed against the peptide are generated from mice immunized with the peptide using standard procedures as referenced herein.

Nucleic acid encoding the monoclonal antibody obtained using the procedures described herein may be cloned and sequenced using technology which is available in the art, and is described, for example, in Wright et al. (1992, Critical Rev. in Immunol. 12(3,4):125-168) and the references cited therein. Further, the antibody of the invention may be  
5 "humanized" using the technology described in Wright et al., (*supra*) and in the references cited therein, and in Gu et al. (1997, Thrombosis and Hematocyst 77(4):755-759).

To generate a phage antibody library, a cDNA library is first obtained from mRNA which is isolated from cells, e.g., the hybridoma, which express the desired peptide to be expressed on the phage surface, e.g., the desired antibody. cDNA copies of the mRNA are  
10 produced using reverse transcriptase. cDNA which specifies immunoglobulin fragments are obtained by PCR and the resulting DNA is cloned into a suitable bacteriophage vector to generate a bacteriophage DNA library comprising DNA specifying immunoglobulin genes. The procedures for making a bacteriophage library comprising heterologous DNA are well known in the art and are described, for example, in Sambrook and Russell (2001, Molecular  
15 Cloning: A Laboratory Manual, Cold Spring Harbor, NY).

Bacteriophage which encode the desired antibody, may be engineered such that the peptide is displayed on the surface thereof in such a manner that it is available for binding to its corresponding binding peptide, e.g., the antigen against which the antibody is directed. Thus, when bacteriophage which express a specific antibody are incubated in the presence of  
20 a cell which expresses the corresponding antigen, the bacteriophage will bind to the cell. Bacteriophage which do not express the antibody will not bind to the cell. Such panning techniques are well known in the art and are described for example, in Wright et al., (*supra*).

Processes such as those described above, have been developed for the production of human antibodies using M13 bacteriophage display (Burton et al., 1994, Adv. Immunol.  
25 57:191-280). Essentially, a cDNA library is generated from mRNA obtained from a population of antibody-producing cells. The mRNA encodes rearranged immunoglobulin genes and thus, the cDNA encodes the same. Amplified cDNA is cloned into M13 expression vectors creating a library of phage which express human antibody fragments on their surface. Phage which display the antibody of interest are selected by antigen binding  
30 and are propagated in bacteria to produce soluble human immunoglobulin. Thus, in contrast

to conventional monoclonal antibody synthesis, this procedure immortalizes DNA encoding human immunoglobulin rather than cells which express human immunoglobulin.

Remodeling glycans of antibody molecules

The specific glycosylation of one class of peptides, namely immunoglobulins, has a  
5 particularly important effect on the biological activity of these peptides. The invention should not be construed to be limited solely to immunoglobulins of the IgG class, but should also be construed to include immunoglobulins of the IgA, IgE and IgM classes of antibodies.

Further, the invention should not be construed to be limited solely to any type of traditional antibody structure. Rather, the invention should be construed to include all types  
10 of antibody molecules, including, for example, fragments of antibodies, chimeric antibodies, human antibodies, humanized antibodies, etc.

A typical immunoglobulin molecule comprises an effector portion and an antigen binding portion. For a review of immunoglobulins, see Harlow et al., 1988, *Antibodies: A Laboratory Manual*, Cold Spring Harbor, New York, and Harlow et al., 1999, *Using  
15 Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, NY. The effector portion of the immunoglobulin molecule resides in the Fc portion of the molecule and is responsible in part for efficient binding of the immunoglobulin to its cognate cellular receptor. Improper glycosylation of immunoglobulin molecules particularly in the CH2 domain of the Fc portion of the molecule, affects the biological activity of the  
20 immunoglobulin.

More specifically with respect to the immunoglobulin IgG, IgG effector function is governed in large part by whether or not the IgG contains an N-acetylglucosamine (GlcNAc) residue attached at the 4-O position of the branched mannose of the trimannosyl core of the N-glycan at Asparagine (Asn) 297 in the CH2 domain of the IgG molecule. This residue is known as a "bisecting GlcNAc." The purpose of adding bisecting GlcNAc to the N-glycan chains of a natural or recombinant IgG molecule or a IgG-Fc-containing chimeric construct is to optimize Fc immune effector function of the Fc portion of the molecule. Such effector functions may include antibody-dependent cellular cytotoxicity (ADCC) and any other biological effects that require efficient binding to Fc<sub>y</sub>R receptors, and binding to the C1 component of complement. The importance of bisecting GlcNAc for achieving maximum  
30

immune effector function of IgG molecules has been described (Lifely et al., 1995, Glycobiology 5 (8): 813-822; Jeffris et al., 1990, Biochem. J. 268 (3): 529-537).

The glycans found at the N-glycosylation site at Asn 297 in the CH2 domain of IgG molecules have been structurally characterized for IgG molecules found circulating in human  
5 and animal blood plasma, IgG produced by myeloma cells, hybridoma cells, and a variety of transfected immortalized mammalian and insect cell lines. In all cases the N-glycan is either a high mannose chain or a complete (Man<sub>3</sub>, GlcNAc<sub>4</sub>, Gal<sub>2</sub>, NeuAc<sub>2</sub>, Fuc<sub>1</sub>) or variably incomplete biantennary chain with or without bisecting GlcNAc (Raju et al., 2000, Glycobiology 10 (5): 477-486; Jeffris et al., 1998, Immunological Rev. 163L59-76; Lerouge et al., 1998, Plant Mol. Biol. 38: 31-48; James et al., 1995, Biotechnology 13: 592-596).

The present invention provides an *in vitro* customized glycosylated immunoglobulin molecule. The immunoglobulin molecule may be any immunoglobulin molecule, including, but not limited to, a monoclonal antibody, a synthetic antibody, a chimeric antibody, a humanized antibody, and the like. Specific methods of generating antibody molecules and  
15 their characterization are disclosed elsewhere herein. Preferably, the immunoglobulin is IgG, and more preferably, the IgG is a humanized or human IgG, most preferably, IgG1.

The present invention specifically contemplates using  $\beta$ 1,4-mannosyl-glycopeptide  $\beta$ 1,4-N-acetylglucosaminyltransferase, GnT-III: EC2.4.1.144 as an *in vitro* reagent to glycosidically link N-acetylglucosamine (GlcNAc) onto the 4-O position of the branched mannose of the trimannosyl core of the N-glycan at Asn 297 in the CH2 domain of an IgG molecule. However, as will be appreciated from the disclosure provided herein, the invention should not be construed to solely include the use of this enzyme to provide a bisecting GlcNAc to an immunoglobulin molecule. Rather, it has been discovered that it is possible to modulate the glycosylation pattern of an antibody molecule such that the antibody molecule  
20 has enhanced biological activity, i.e., effector function, in addition to potential enhancement of other properties, e.g., stability, and the like.

There is provided in the present invention a general method for removing fucose molecules from the Asn(297) N-linked glycan for the purpose of enhancing binding to Fc-gammaRIIA, and enhanced antibody-dependent cellular cytotoxicity (see, Shields et al.,  
30 2002, J. Biol. Chem. 277:26733-26740). The method entails contacting the antibody molecule with a fucosidase appropriate for the linkage of the fucose molecule(s) on the

antibody glycan(s). Alternately, the recombinant antibody can be expressed in cells that do express fucosyltransferases, such as the Lec13 variant of CHO cells. The removal of fucose from the glycan(s) of the antibody can be done alone, or in conjunction with other methods to remodel the glycans, such as adding a bisecting GlcNAc. Expression of antibodies in cells lacking GnT-I may also result in Fc glycans lacking core fucose, which can be further modified by the present invention.

There is provided in the present invention a general method for introducing a bisecting GlcNAc for the purpose of enhancing Fc immune effector function in any preparation of IgG molecules containing N-linked oligosaccharides in the CH2 domain, typically at Asn 297. The method requires that the population of IgG molecules is brought to a state of glycosylation such that the glycan chain is an acceptor for GnT-III. This is accomplished in any one of three ways: 1) by selection or genetic manipulation of a host expression system that secretes IgG with N-glycan chains that are substrates for GnT-III; 2) by treatment of a population of IgG glycoforms with exoglycosidases such that the glycan structure(s) remaining after exoglycosidase treatment is an acceptor for GnT-III; 3) some combination of host selection and exoglycosidase treatment as in 1) and 2) above plus successive additions of GlcNAc by GnT-I and GnT-II to create an acceptor for GnT-III.

For example, IgG obtained from chicken plasma contains primarily high mannose chains and would require digestion with one or more  $\alpha$ -mannosidases to create a substrate for addition of GlcNAc to the  $\alpha$ 1,3 Mannose branch of the trimannosyl core by GnT-I. This substrate could be the elemental trimannosyl core, Man3GlcNAc2. Treatment of this core structure sequentially with GnT-I followed by GnT-II followed by GnT-III using UDP-GlcNAc as a sugar donor would create Man3GlcNAc5 as shown in Figure 2. Optionally, this structure can then be extended by treatment with  $\beta$ 1,4 galactosyltransferase. If required, the galactosylated oligosaccharide can be further extended using  $\alpha$ 2,3- or  $\alpha$ 2,6-sialyltransferase to achieve a completed biantennary structure. Using this method biantennary glycan chains can be remodeled as required for the optimal Fc immune effector function of any therapeutic IgG under development (Figure 4).

Alternatively, IgG molecules found in the plasma of most animals or IgG which is secreted as a recombinant product by most animal cells or by transgenic animals typically include a spectrum of biantennary glycoforms including complete (NeuAc2, Gal2, GlcNAc4,

Man3, ±Fuc1) (Figure 4) and variably incomplete forms, with or without bisecting GlcNAc (Raju et al., 2000, Glycobiology 10 (5): 477-486; Jeffris et al., 1998, Immunological Rev. 163: 59-76). To ensure that bisecting GlcNAc is present in the entire population of immunoglobulin molecules so produced, the mixture of molecules can be treated with the 5 following exoglycosidases, successively or in a mixture: neuraminidase,  $\beta$ -galactosidase,  $\beta$ -glucosaminidase,  $\alpha$ -fucosidase. The resulting trimannosyl core can then be remodeled using glycosyltransferases as noted above.

In addition, IgG secreted by transgenic animals or stored as "plantibodies" by transgenic plants have been characterized. An IgG molecule produced in a transgenic plant 10 having N-glycans that contain  $\beta$ 1,2 linked xylose and/or  $\alpha$ 1,3 linked fucose can be treated with exoglycosidases to remove those residues, in addition to the above described exoglycosidases in order to create the trimannosyl core or a Man3GlcNAc4 structure, and are then treated with glycosyltransferases to remodel the N-glycan as described above.

The primary novel aspect of the current invention is the application of appropriate 15 glycosyltransferases, with or without prior exoglycosidase treatment, applied in the correct sequence to optimize the effector function of the antibody. In one exemplary embodiment, a bisecting GlcNAc is introduced into the glycans of IgG molecules or other IgG-Fc-chimeric constructs where bisecting GlcNAc is required. In another exemplary embodiment, the core fucose is removed from the glycans of IgG molecules or other IgG-Fc-chimeric 20 constructs.

#### TNF receptor-IgG Fc fusion protein

The nucleotide and amino acid sequences of the 75 kDa human TNF receptor are set forth herein as SEQ ID NO:31 and SEQ ID NO:32, respectively (Figure 71A and 71B, respectively). The amino acid sequences of the light and heavy variable regions of chimeric 25 anti-HER2 are set forth as SEQ ID NO:35 and SEQ ID NO:36, respectively (Figure 72A and 72B, respectively). The amino acid sequences of the light and heavy variable regions of chimeric anti-RSV are set forth as, and SEQ ID NO:38 and SEQ ID NO:37, respectively (Figure 73A and 73B, respectively). The amino acid sequences of the non-human variable regions of anti-TNF are set forth herein as SEQ ID NO:41 and SEQ ID NO:42, respectively 30 (Figure 74A and 74B, respectively). The nucleotide and amino acid sequence of the Fc

portion of human IgG is set forth as SEQ ID NO:49 and SEQ ID NO:50 (Figure 75A and 75B, respectively).

MAb anti-glycoprotein IIb/IIIa

The amino acid sequences of a murine anti-glycoprotein IIb/IIIa antibody variable

- 5 regions are set forth in SEQ ID NO:52 (murine mature variable light chain, Figure 76) and SEQ ID NO: 54 (murine mature variable heavy chain, Figure 77). These murine sequences can be combined with human IgG amino acid sequences SEQ ID NO: 51 (human mature variable light chain, Figure 78), SEQ ID NO: 53 (human mature variable heavy chain, Figure 79), SEQ ID NO: 55 (human light chain, Figure 80) and SEQ ID NO: 56 (human heavy 10 chain, Figure 81) according to the procedures found in U.S. Patent No. 5,777,085 to create a chimeric humanized murine anti-glycoprotein IIb/IIIa antibody. Other anti-glycoprotein IIb/IIIa humanized antibodies are found in U.S. Patent No. 5,877,006. A cell line expressing the anti-glycoprotein IIb/IIIa MAb 7E3 can be commercially obtained from the ATCC (Manassas, VA) as accession no. HB-8832.

15 MAb anti-CD20

The nucleic acid and amino acid sequences of a chimeric anti-CD20 antibody are set forth in SEQ ID NO: 59 (nucleic acid sequence of murine variable region light chain, Figure 82A), SEQ ID NO: 60 (amino acid sequence of murine variable region light chain, Figure 82B), SEQ ID NO: 61 (nucleic acid sequence of murine variable region heavy chain, Figure 20 83A) and SEQ ID NO: 62 (amino acid sequence of murine variable region heavy chain, Figure 83B). In order to humanize a murine antibody, the TCAE 8 (SEQ ID NO: 57, Figure 84A – 84E), which contains the human IgG heavy and light constant domains, may be conveniently used. By cloning the above murine variable region encoding DNA into the TCAE 8 vector according to instructions given in U.S. Patent No. 5,736,137, a vector is 25 created (SEQ ID NO: 58, Figure 85A – 85E) which when transformed into a mammalian cell line, expresses a chimeric anti-CD20 antibody. Other humanized anti-CD20 antibodies are found in U.S. Patent No. 6,120,767. A cell line expressing the anti-CD20 MAb C273 can be commercially obtained from the ATCC (Manassas, VA) as accession no. HB-9303.

The skilled artisan will readily appreciate that the sequences set forth herein are not

- 30 exhaustive, but are rather examples of the variable regions, receptors, and other binding moieties of chimeric antibodies. Further, methods to construct chimeric or "humanized"

antibodies are well known in the art, and are described in, for example, U.S. Patent No. 6,329,511 and U.S. Patent No. 6,210,671. Coupled with the present disclosure and methods well known throughout the art, the skilled artisan will recognize that the present invention is not limited to the sequences disclosed herein.

5       The expression of a chimeric antibody is well known in the art, and is described in detail in, for example, U.S. Patent No. 6,329,511. Expression systems can be prokaryotic, eukaryotic, and the like. Further, the expression of chimeric antibodies in insect cells using a baculovirus expression system is described in Putlitz et al. (1990, Bio/Technology 8:651-654). Additionally, methods of expressing a nucleic acid encoding a fusion or chimeric  
10 protein are well known in the art, and are described in, for example, Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York) and Ausubel et al. (1997, Current Protocols in Molecular Biology, Green & Wiley, New York).

15      Determining the function and biological activity of a chimeric antibody produced according to the methods of the present invention is a similarly basic operation for one of skill in the art. Methods for determining the affinity of an antibody by competition assays are detailed in Berzofsky (J. A. Berzofsky and I. J. Berkower, 1984, in Fundamental Immunology (ed. W. E. Paul), Raven Press (New York), 595). Briefly, the affinity of the chimeric antibody is compared to that of the monoclonal antibody from which it was derived  
20 using a radio-iodinated monoclonal antibody.

#### VII. Pharmaceutical Compositions

In another aspect, the invention provides a pharmaceutical composition. The pharmaceutical composition includes a pharmaceutically acceptable diluent and a covalent  
25 conjugate between a non-naturally-occurring, water-soluble polymer, therapeutic moiety or biomolecule and a glycosylated or non-glycosylated peptide. The polymer, therapeutic moiety or biomolecule is conjugated to the peptide via an intact glycosyl linking group interposed between and covalently linked to both the peptide and the polymer, therapeutic moiety or biomolecule.

30      Pharmaceutical compositions of the invention are suitable for use in a variety of drug delivery systems. Suitable formulations for use in the present invention are found in

*Remington's Pharmaceutical Sciences*, Mace Publishing Company, Philadelphia, PA, 17th ed. (1985). For a brief review of methods for drug delivery, see, Langer, *Science* 249:1527-1533 (1990).

The pharmaceutical compositions may be formulated for any appropriate manner of administration, including for example, topical, oral, nasal, intravenous, intracranial, intraperitoneal, subcutaneous or intramuscular administration. For parenteral administration, such as subcutaneous injection, the carrier preferably comprises water, saline, alcohol, a fat, a wax or a buffer. For oral administration, any of the above carriers or a solid carrier, such as mannitol, lactose, starch, magnesium stearate, sodium saccharine, talcum, cellulose, glucose, sucrose, and magnesium carbonate, may be employed. Biodegradable microspheres (e.g., polylactate polyglycolate) may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268 and 5,075,109.

Commonly, the pharmaceutical compositions are administered parenterally, e.g., intravenously. Thus, the invention provides compositions for parenteral administration which comprise the compound dissolved or suspended in an acceptable carrier, preferably an aqueous carrier, e.g., water, buffered water, saline, PBS and the like. The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as pH adjusting and buffering agents, tonicity adjusting agents, wetting agents, detergents and the like.

These compositions may be sterilized by conventional sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile aqueous carrier prior to administration. The pH of the preparations typically will be between 3 and 11, more preferably from 5 to 9 and most preferably from 7 and 8.

In some embodiments the peptides of the invention can be incorporated into liposomes formed from standard vesicle-forming lipids. A variety of methods are available for preparing liposomes, as described in, e.g., Szoka *et al.*, *Ann. Rev. Biophys. Bioeng.* 9: 467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728 and 4,837,028. The targeting of liposomes using a variety of targeting agents (e.g., the sialyl galactosides of the invention) is well known in the art (see, e.g., U.S. Patent Nos. 4,957,773 and 4,603,044).

Standard methods for coupling targeting agents to liposomes can be used. These methods generally involve incorporation into liposomes of lipid components, such as phosphatidylethanolamine, which can be activated for attachment of targeting agents, or derivatized lipophilic compounds, such as lipid-derivatized peptides of the invention.

- 5 Targeting mechanisms generally require that the targeting agents be positioned on the surface of the liposome in such a manner that the target moieties are available for interaction with the target, for example, a cell surface receptor. The carbohydrates of the invention may be attached to a lipid molecule before the liposome is formed using methods known to those of skill in the art (e.g., alkylation or acylation of a hydroxyl group present on the
- 10 carbohydrate with a long chain alkyl halide or with a fatty acid, respectively). Alternatively, the liposome may be fashioned in such a way that a connector portion is first incorporated into the membrane at the time of forming the membrane. The connector portion must have a lipophilic portion, which is firmly embedded and anchored in the membrane. It must also have a reactive portion, which is chemically available on the aqueous surface of the liposome.
- 15 The reactive portion is selected so that it will be chemically suitable to form a stable chemical bond with the targeting agent or carbohydrate, which is added later. In some cases it is possible to attach the target agent to the connector molecule directly, but in most instances it is more suitable to use a third molecule to act as a chemical bridge, thus linking the connector molecule which is in the membrane with the target agent or carbohydrate which is extended,
- 20 three dimensionally, off of the vesicle surface. The dosage ranges for the administration of the peptides of the invention are those large enough to produce the desired effect in which the symptoms of the immune response show some degree of suppression. The dosage should not be so large as to cause adverse side effects. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the animal and can be determined by one of skill in
- 25 the art. The dosage can be adjusted by the individual physician in the event of any counterindications.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved by the use of polymers to conjugate, complex or adsorb the peptide. The controlled delivery may be exercised by selecting appropriate macromolecules (for example, polyesters, polyamino carboxymethylcellulose, and protamine sulfate) and the concentration of macromolecules as well as the methods of

incorporation in order to control release. Another possible method to control the duration of action by controlled release preparations is to incorporate the peptide into particles of a polymeric material such as polyesters, polyamino acids, hydrogels, poly (lactic acid) or ethylene vinylacetate copolymers.

5 In order to protect peptides from binding with plasma proteins, it is preferred that the peptides be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly (methymethacrylate) microcapsules, respectively, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and  
10 nanocapsules or in macroemulsions. Such teachings are disclosed in Remington's Pharmaceutical Sciences (16th Ed., A. Oslo, ed., Mack, Easton, Pa., 1980).

The peptides of the invention are well suited for use in targetable drug delivery systems such as synthetic or natural polymers in the form of macromolecular complexes, nanocapsules, microspheres, or beads, and lipid-based systems including oil-in-water  
15 emulsions, micelles, mixed micelles, liposomes, and resealed erythrocytes. These systems are known collectively as colloidal drug delivery systems. Typically, such colloidal particles containing the dispersed peptides are about 50 nm-2 µm in diameter. The size of the colloidal particles allows them to be administered intravenously such as by injection, or as an aerosol. Materials used in the preparation of colloidal systems are typically sterilizable via filter  
20 sterilization, nontoxic, and biodegradable, for example albumin, ethylcellulose, casein, gelatin, lecithin, phospholipids, and soybean oil. Polymeric colloidal systems are prepared by a process similar to the coacervation of microencapsulation.

In an exemplary embodiment, the peptides are components of a liposome, used as a targeted delivery system. When phospholipids are gently dispersed in aqueous media, they  
25 swell, hydrate, and spontaneously form multilamellar concentric bilayer vesicles with layers of aqueous media separating the lipid bilayer. Such systems are usually referred to as multilamellar liposomes or multilamellar vesicles (MLVs) and have diameters ranging from about 100 nm to about 4 µm. When MLVs are sonicated, small unilamellar vesicles (SUVS) with diameters in the range of from about 20 to about 50 nm are formed, which contain an  
30 aqueous solution in the core of the SUV.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and 5 are saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine, and distearoylphosphatidylcholine.

In preparing liposomes containing the peptides of the invention, such variables as the efficiency of peptide encapsulation, lability of the peptide, homogeneity and size of the resulting population of liposomes, peptide-to-lipid ratio, permeability instability of the 10 preparation, and pharmaceutical acceptability of the formulation should be considered.

Szoka, et al, *Annual Review of Biophysics and Bioengineering*, 9: 467 (1980); Deamer, et al., in *LIPOSOMES*, Marcel Dekker, New York, 1983, 27: Hope, et al., *Chem. Phys. Lipids*, 40: 89 (1986).

The targeted delivery system containing the peptides of the invention may be 15 administered in a variety of ways to a host, particularly a mammalian host, such as intravenously, intramuscularly, subcutaneously, intra-peritoneally, intravascularly, topically, intracavarily, transdermally, intranasally, and by inhalation. The concentration of the peptides will vary upon the particular application, the nature of the disease, the frequency of administration, or the like. The targeted delivery system-encapsulated peptide may be 20 provided in a formulation comprising other compounds as appropriate and an aqueous physiologically acceptable medium, for example, saline, phosphate buffered saline, or the like.

The compounds prepared by the methods of the invention may also find use as 25 diagnostic reagents. For example, labeled compounds can be used to locate areas of inflammation or tumor metastasis in a patient suspected of having an inflammation. For this use, the compounds can be labeled with  $^{125}\text{I}$ ,  $^{14}\text{C}$ , or tritium.

#### EXPERIMENTAL EXAMPLES

The invention is now described with reference to the following Examples. These 30 Examples are provided for the purpose of illustration only and the invention should in no way

be construed as being limited to these Examples, but rather should be construed to encompass any and all variations which become evident as a result of the teaching provided herein.

#### A. Glycosylation

5 The materials and methods used in the experiments presented in this Example are now described.

##### 1. Sialylation and Fucosylation of TP10

This example sets forth the preparation of TP10 with sialyl Lewis X moieties and analysis of enhanced biological activity.

10 Interrupting blood flow to the brain, even for a short time, can trigger inflammatory events within the cerebral microvasculature that can exacerbate cerebral tissue damage. The tissue damage that accrues is amplified by activation of both inflammation and coagulation cascades. In a murine model of stroke, increased expression of P-selectin and ICAM-1 promotes leukocyte recruitment. sCR1 is recombinant form of the extracellular domain of

15 Complement Receptor-1 (CR-1). sCR-1 is a potent inhibitor of complement activation. sCR1sLe<sup>X</sup> (CD20) is an alternately glycosylated form of sCR1 that is alternately glycosylated to display sialylated Lewis<sup>X</sup> antigen. Previously, sCR-1sLeX that was expressed and glycosylated *in vivo* in engineered Lec11 CHO cells was found to correctly localize to ischemic cerebral microvessels and C1q-expressing neurons, thus inhibiting 20 neutrophil and platelet accumulation and reducing cerebral infarct volumes (Huang et al., 1999, Science 285:595-599). In the present example, sCR1sLe<sup>X</sup> which was prepared *in vitro* by remodeling of glycans, exhibited enhanced biological activity similar to that of sCRsLe<sup>X</sup> glycosylated *in vivo*.

25 The TP10 peptide was expressed in DUK B11 CHO cells. This CHO cell line produces the TP10 peptide with the typical CHO cell glycosylation, with many but not all glycans capped with sialic acid.

30 **Sialylation of 66 mg of TP10.** TP10 (2.5 mg/mL), CMPSA (5 mM), and ST3Gal3 (0.1 U/mL) were incubated at 32°C in 50 mM Tris, 0.15M NaCl, 0.05% sodium azide, pH 7.2 for 48 hours. Radiolabelled CMP sialic acid was added to a small aliquot to monitor incorporation. TP10 was separated from nucleotide sugar by SEC HPLC. Samples analyzed at 24 hours and 48 hours demonstrated that the reaction was completed after 24 hours. The

reaction mixture was then frozen. The reaction products were subjected to Fluorophore Assisted Carbohydrate Electrophoresis (FACE<sup>®</sup>; Glyko, Inc, Novato CA) analysis (Figure 86).

**Pharmacokinetic studies.** Rats were purchased with a jugular vein cannula.

- 10 5 mg/kg of either the pre-sialylation or post-sialylation TP10 peptide was given by tail vein injection to three rats for each treatment (n=3). Fourteen blood samples were taken from 0 to 50 hours. The concentration in the blood of post-sialylation TP10 peptide was higher than that of pre-sialylation TP10 at every time point past 0 hour (Figure 87). Sialic acid addition doubled the area under the plasma concentration-time curve (AUC) of the pharmacokinetic 10 curve as compared to the starting material (Figure 88).

15 **Fucosylation of sialylated TP10.** 10 mL (25 mg TP10) of the above sialylation mix was thawed, and GDP-fucose was added to 5 mM, MnCl<sub>2</sub> to 5 mM, and FTVI (fucosyltransferase VI) to 0.05 U/mL. The reaction was incubated at 32°C for 48 hours. The reaction products were subjected to Fluorophore Assisted Carbohydrate Electrophoresis 15 (FACE<sup>®</sup>; Glyko, Inc, Novato CA) analysis (Figure 89). To a small aliquot, radiolabelled GDP-fucose was added to monitor incorporation. TP10 was separated from nucleotide sugar by SEC HPLC. Samples analyzed at 24 hours and 48 hours demonstrated that the reaction was completed at 24 hours. An *in vitro* assay measuring binding to E-selectin indicate that fucose addition can produce a biologically-active E-selectin ligand (Figure 90).

20

## 2. Sialylation of Recombinant Glycoproteins

This example sets forth the preparation of sialylated forms of several recombinant peptides.

**Sialylation of Recombinant Glycoproteins Using ST3Gal III.** Several

- 25 glycoproteins were examined for their ability to be sialylated by recombinant rat ST3Gal III. For each of these glycoproteins, sialylation will be a valuable process step in the development of the respective glycoproteins as commercial products.

30 **Reaction Conditions.** Reaction conditions were as summarized in Table 9. The sialyltransferase reactions were carried out for 24 hour at a temperature between room temperature and 37°. The extent of sialylation was established by determining the amount of

<sup>14</sup>C-NeuAc incorporated into glycoprotein-linked oligosaccharides. See Table 9 for the reaction conditions for each protein.

Table 9. Reaction conditions.

Protein	Source	Protein Total (mg)	Protein Conc. (mg/ml)	ST (mU/mL)	ST/Protein (mU/mg)	CMP-NeuAc of "cycle" <sup>1</sup>
ATIII	Genzyme Transgenics	8.6	4.3	210	48	cycle
ATIII	Genzyme Transgenics	860	403	53	12	cycle
Asialo-fetuin	Sigma	0.4	105	20	13	10 mM
asilao-AAAT	PPL	0.4	0.5	20	20	20 mM

<sup>1</sup> "Cycle" refers to generation of CMP-NeuAc "in situ" enzymatically using standard conditions as described in specification (20 mM NeuAc and 2 mM CMP). The buffer was 5 0.1 M HEPES, pH 7.5.

The results presented in Table 10 demonstrate that a remarkable extent of sialylation was achieved in every case, despite low levels of enzyme used. Essentially, complete 10 sialylation was obtained, based on the estimate of available terminal galactose. Table 10 shows the results of the sialylation reactions. The amount of enzyme used per mg of protein (mU/mg) as a basis of comparison for the various studies. In several of the examples shown, only 7-13 mU ST3Gal III per mg of protein was required to give essentially complete sialylation after 24 hours.

Table 10. Analytical results

Protein	Source	Terminal Gal <sup>1</sup> mol/mol	NeuAc Incorp. <sup>2</sup> mol/mol	% Rxn <sup>3</sup>	Other characterization
ATIII <sup>4</sup>	Genzyme Transgenics	102	104	117	None
ATIII <sup>4</sup>	Genzyme Transgenics	102	1.3	108	SDS-gels; protein purity FACs: carbohydrate glycoforms None
Asialo-fetuin	Sigma	802	905	116	
asilao-AAAT <sup>5</sup>	PPL	7	7.0	100	SDS-gels; protein purity

<sup>1</sup> Terminal (exposed) Gal content on N-linked oligosaccharides determined by supplier, or from literature values (fetuin, asialo-AAAT).

<sup>2</sup> NeuAc incorporated determined by incorporation of 14C-NeuAc after separation from free radiolabeled precursors by gel filtration.

<sup>3</sup> The % Rxn refers to % completion of the reaction based on the terminal Gal content as a theoretical maximum.

<sup>4</sup> Antithrombin III.

<sup>5</sup>  $\alpha$ 1 Antitrypsin.

These results are in marked contrast to those reported in detailed studies with bovine ST6Gal I where less than 50 mU/mg protein gave less than 50% sialylation, and 1070 mU/mg protein gave approximately 85-90% sialylation in 24 hours. Paulson et al. (1977) J. Biol. Chem. 252: 2363-2371; Paulson et al. (1978) J. Biol. Chem. 253: 5617-5624. A study of rat  $\alpha$ 2,3 and  $\alpha$ 2,6 sialyltransferases by another group revealed that complete sialylation of asialo-AGP required enzyme concentrations of 150-250 mU/mg protein (Weinstein et al. (1982) J. Biol. Chem. 257: 13845-13853). These earlier studies taken together suggested that the ST6Gal I sialyltransferase requires greater than 50 mU/mg and up to 150 mU/mg to achieve complete sialylation.

This Example demonstrates that sialylation of recombinant glycoproteins using the ST3 Gal III sialyltransferase required much less enzyme than expected. For a one kilogram scale reaction, approximately 7,000 units of the ST3Gal III sialyltransferase would be needed, instead of 100,000-150,000 units that earlier studies indicated. Purification of these enzymes from natural sources is prohibitive, with yields of only 1-10 units for a large scale preparation after 1-2 months work. Assuming that both the ST6Gal I and ST3Gal III sialyltransferases are produced as recombinant sialyltransferases, with equal levels of expression of the two enzymes being achieved, a fermentation scale 14-21 times greater (or more) would be required for the ST6Gal I sialyltransferase relative to the ST3Gal III sialyltransferase. For the ST6Gal I sialyltransferase, expression levels of 0.3 U/l in yeast has been reported (Borsig et al. (1995) Biochem. Biophys. Res. Commun. 210: 14-20). Expression levels of 1000 U/liter of the ST3 Gal III sialyltransferase have been achieved in *Aspergillus niger*. At current levels of expression 300-450,000 liters of yeast fermentation would be required to produce sufficient enzyme for sialylation of 1 kg of glycoprotein using the ST6Gal I sialyltransferase. In contrast, less than 10 liter fermentation of *Aspergillus niger* would be required for sialylation of 1 kg of glycoprotein using the ST3Gal III sialyltransferase. Thus, the fermentation capacity required to produce the ST3Gal III

sialyltransferase for a large scale sialylation reaction would be 10-100 fold less than that required for producing the ST6Gal I; the cost of producing the sialyltransferase would be reduced proportionately.

5           3. Fucosylation to create Sialyl Lewis X

This example sets forth the preparation of Tissue Type Plasminogen Activator (TPA) with N-linked sialyl Lewis X antigen.

Sialylation. TPA expressed in mammalian cells will often contain a majority of the glycans terminating in sialic acid, but to ensure complete sialylation, it would be beneficial to 10 first perform an *in vitro* sialylation. TPA in a suitable buffer (most preferably between pH 5.5 and 9, for example Tris buffered saline, pH 7.2) is incubated with CMP sialic acid and sialyltransferase for a time sufficient to convert any glycans lacking sialic acid to sialylated species. Typical conditions would be 1 mg/mL TPA, 3 mM CMP sialic acid, 0.02 U/mL ST3Gal3, 32°C for 24 hours. Microbial growth can be halted either by sterile filtration or the 15 inclusion of 0.02% sodium azide. The TPA concentration is most preferably in the range 0.1 mg/mL up to the solubility limit of the peptide. The concentration of CMP-SA should be sufficient for there to be excess over the available sites, and might range from 50 µM up to 50 mM, and the temperature from 2°C up to 40°C. The time required for complete reaction will depend on the temperature, the relative amounts of enzyme to acceptor substrate, the donor 20 substrate concentration, and the pH. Other sialyltransferases that may be capable of adding sialic acid in 2,3 linkage include ST3Gal4; microbial transferases could also be used.

Fucosylation. Typical conditions for fucosylation would be 1 mg/mL TPA, 3 mM GDP-fucose, 0.02 U/mL FTVI, 5 mM MnCl<sub>2</sub>, 32°C for 24H in Tris buffered saline.

Microbial growth can be halted either by sterile filtration or the inclusion of 0.02% sodium 25 azide. The TPA concentration is most preferably in the range 0.1 mg/mL up to the solubility limit of the peptide. The concentration of GDP-fucose should be sufficient for there to be excess over the available sites, and might range from 50 µM up to 50 mM, and the temperature from 2°C up to 40°C. The time required for complete reaction will depend on the temperature, the relative amounts of enzyme to acceptor substrate, the donor substrate 30 concentration, and the pH. Other fucosyltransferases that may be capable of making sialyl Lewis x include FTVII, FTV, FTIII, as well as microbial transferases could also be used.

**4. Trimming of high mannose to tri-mannose core structure: Tissue-type Plasminogen Activator produced in CHO**

This example sets forth the preparation of Tissue-type Plasminogen Activator with a  
5 trimannose core by trimming back from a high mannose glycan.

Tissue-type plasminogen activator (TPA) is currently produced in Chinese Hamster Ovary (CHO) cells and contains a low amount of high mannose N-linked oligosaccharide. The mannoses can be trimmed down using a variety of the specific mannosidases. The first step is to generate Man<sub>5</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1) from Man<sub>9</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1). This can be done  
10 using mannosidase I. Then either GlcNAcT1 (GlcNAc transferase I) is used to make GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1) or Mannosidase III is used to make Man<sub>3</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1). From Man<sub>3</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1), GlcNAc<sub>1</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1) can be produced using GlcNAcT1 or from GlcNAc<sub>1</sub>Man<sub>5</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1), GlcNAc<sub>1</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1) can be produced using Mannosidase II. GlcNAc<sub>1</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1) is then converted into  
15 GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>(Fuc<sub>0</sub>-1) using GlcNAc Transferase II (GlcNAcTII). The two terminal GlcNAc residues are then galactosylated using GalTI and then sialylated with SA-PEG using ST3GalIII.

Conversely, TPA can be produced in yeast or fungal systems. Similar processing would be required for fungal derived material.

20

**5. Addition of GlcNAc to EPO**

This example sets forth the addition of a GlcNAc residue on to a tri-mannosyl core.

**Addition of GlcNAc to EPO.** EPO was expressed in SF-9 insect cells and purified (Protein Sciences, Meriden, CT). A 100% conversion from the tri-mannosyl glycoform of  
25 Epo to the "tri-mannosyl core + 2 GlcNAc" (Peak 1, P1 in Figure 91) was achieved in 24 hours of incubation at 32°C with 100mU/ml of GlcNAcT-I and 100mU/ml of GlcNAcT-II in the following reaction final concentrations:

100mM MES pH 6.5, or 100mM Tris pH 7.5

5mM UDP-GlcNAc

30 20mM MnCl<sub>2</sub>

100mU/ml GlcNAcT-I

100mU/ml GlcNAcT-II  
1 mg/ml EPO (purified, expressed in Sf9 cells,  
purchased from Protein Sciences).

**Analysis of glycoforms.** This assay is a slight modification on K-R Anumula and ST

- 5 Dhume, Glycobiology 8 (1998) 685-69. N-glycanase (PNGase) released N-glycans were reductively labeled with anthranilic acid. The reductively-aminated N-glycans were injected onto a Shodex Asahipak NH2P-50 4D amino column (4.6 mm x 150 mm). Two solvents were used for the separation: A) 5% (v/v) acetic acid, 1% tetrahydrofuran, and 3% triethylamine in water, and B) 2% acetic acid and 1% tetrahydrofuran in acetonitrile. The 10 column was then eluted isocratically with 70% B for 2.5 minutes, followed by a linear gradient over a period of 97.5 minutes going from 70 to 5% B and a final isocratic elution with 5% B for 15 minutes. Eluted peaks were detected using fluorescence detection with an excitation of 230 nm and emission wavelength of 420 nm.

Under these conditions, the trimannosyl core had a retention time of 22.3 minutes, and 15 the product of the GnT reaction has a retention time of 26.3 minutes. The starting material was exclusively trimannosyl core with core GlcNAc (Figure 91).

6. Remodeling high mannose N-glycans to hybrid and complex N-glycans:  
Bovine pancreatic RNase

20 This example sets forth the preparation of bovine pancreas RNase with hybrid or complex N-glycans. The high mannose N-linked glycans of the RNase are enzymatically digested and elaborated on to create hybrid N-linked glycans. Additionally, the high mannose N-linked glycans of the RNase are enzymatically digested and elaborated on to create complex N-linked glycans.

25 High mannose structures of *N*-linked oligosaccharides in glycopeptides can be modified to hybrid or complex forms using the combination of  $\alpha$ -mannosidases and glycosyltransferases. This example summarizes the results in such efforts using a simple *N*-Glycan as a model substrate.

30 Ribonuclease B (RNaseB) purified from bovine pancreas (Sigma) is a glycopptide consisting of 124 amino acid residues. It has a single potential *N*-glycosylation site modified with high mannose structures. Due to its simplicity and low molecular weight (13.7 kDa to

15.5 kDa), ribonuclease B is a good candidate to demonstrate the feasibility of the *N*-Glycan remodeling from high mannose structures to hybrid or complex *N*-linked oligosaccharides. The MALDI-TOF spectrum of RNaseB and HPLC profile for the oligosaccharides cleaved from RNaseB by N-Glycanase (Figure 92) indicated that, other than a small portion of the 5 non-modified peptide, the majority of *N*-glycosylation sites of the peptide are modified with high mannose oligosaccharides consisting of 5 to 9 mannose residues.

**Conversion of high mannose *N*-Glycans to hybrid *N*-Glycans.** High mannose *N*-Glycans were converted to hybrid *N*-Glycans using the combination of  $\alpha$ 1,2-mannosidase, GlcNAcT-I ( $\beta$ 1,2-*N*-acetyl glucosaminyl transferase), GalT-I ( $\beta$ 1,4-galactosyltransferase) and 10  $\alpha$ 2,3-sialyltransferase /or  $\alpha$ 2,6-sialyltransferase as shown in Figure 93.

As an example, high mannose structures in RNaseB were successfully converted to hybrid structures.

Man<sub>5</sub>GlcNAc<sub>2</sub>-R was obtained from Man<sub>5,9</sub>GlcNAc<sub>2</sub>-R catalyzed by a single  $\alpha$ 1,2-mannosidase cloned from *Trichoderma reesei* (Figure 94). RNase B (1 g, about 67  $\mu$ mol) 15 was incubated at 30°C for 45 h with 15 mU of the recombinant *T. reesei*  $\alpha$ 1,2-mannosidase in MES buffer (50 mM, pH 6.5) in a total volume of 10 mL. Man<sub>5,9</sub>GlcNAc<sub>2</sub>-protein structures have been successfully converted to Man<sub>5</sub>GlcNAc<sub>2</sub>-protein with high efficiency by the recombinant mannosidase.

Alternately, Man<sub>5</sub>GlcNAc<sub>2</sub>-R was obtained from Man<sub>5,9</sub>GlcNAc<sub>2</sub>-R catalyzed by a 20 single  $\alpha$ 1,2-mannosidase purified from *Aspergillus saitoi* (Figure 95). RNase B (40  $\mu$ g, about 2.7 nmol) was incubated at 37°C for 42.5 h with 25  $\mu$ U of the commercial *A. saitoi*  $\alpha$ 1,2-mannosidase (Glyko or CalBioChem) in NaOAC buffer (100 mM, pH 5.0) in a total volume of 20  $\mu$ L. Man<sub>5,9</sub>GlcNAc<sub>2</sub>-protein structures were successfully converted to Man<sub>5</sub>GlcNAc<sub>2</sub>-protein by the commercially available mannosidase. However, a new peak corresponding to the GlcNAc-protein appears in the spectrum, indicating the possible contamination of 25 endoglycosidase H in the preparation. Although several mammalian alpha-mannosidases were required to achieve this step, the fungal  $\alpha$ 1,2-mannosidase was very efficient to remove all  $\alpha$ 1,2-linked mannose residues.

GlcNAcT-I then added a GlcNAc residue to the Man<sub>5</sub>GlcNAc<sub>2</sub>-R (Figure 96). The 30 reaction mixture after the *T. reesei*  $\alpha$ 1,2-mannosidase reaction containing RNase B (600  $\mu$ g,

about 40 nmol) was incubated with non-purified recombinant GlcNAcT-I (34 mU) in MES buffer (50 mM, pH 6.5) containing MnCl<sub>2</sub> (20 mM) and UDP-GlcNAc (5 mM) in a total volume of 400 µl. at 37°C for 42 h. A GlcNAc residue was quantitatively added to Man<sub>5</sub>GlcNAc<sub>2</sub>-protein by the recombinant GlcNAcT-I.

5       A Gal residue was then added using GaIT 1 (Figure 97). The reaction mixture after the GnT-I reaction containing RNase B (120 µg, about 8 nmol) was incubated at 37°C for 20 h with 3.3 mU of the recombinant GaIT-1 in Tris-HCl buffer (100 mM, pH 7.3) containing UDP-Gal (7.5 mM) and MnCl<sub>2</sub> (20 mM) in a total volume of 100 µl. A Gal residue was added to about 98% of the GlcNAc-Man<sub>5</sub>GlcNAc<sub>2</sub>-protein by the recombinant GaIT 1.

10       The next step was the addition of a sialic acid using an α2,3-sialyltransferase or an α2,6-sialyltransferase (Figure 98). As an example, ST3Gal III, an α2,3-sialyltransferase was used. The reaction mixture after the GaIT-1 reaction containing RNase B (13 µg, about 0.87 nmol) was incubated at 37°C for 16 h with 8.9 mU of recombinant ST3Gal III in Tris-HCl buffer (100 mM, pH 7.3) containing CMP-Sialic acid (5 mM) and MnCl<sub>2</sub> (20 mM) in a total volume of 20 µl. A sialic acid residue was added to about 90% of the Gal-GlcNAc-Man<sub>5</sub>GlcNAc<sub>2</sub>-protein by recombinant ST3Gal III using CMP-SA as the donor. The yield can be further improved by adjusting the reaction conditions.

15       For convenience, no purification or dialysis step was required after each reaction described above. More interesting, GaIT 1 and ST3Gal III can be combined in a one-pot reaction. Similar yields were obtained as compared with the separate reactions. The reaction mixture after the GlcNAcT-I reaction containing RNase B (60 µg, about 4 nmol) was incubated at 37°C for 20 h with 1.7 mU of recombinant GaIT 1, 9.8 mU of recombinant ST3Gal III in Tris-HCl buffer (100 mM, pH 7.3) containing UDP-Gal (7.5 mM), CMP-sialic acid (5 mM) and MnCl<sub>2</sub> (20 mM) in a total volume of 60 µl.

20       As shown in Figure 99, SA-PEG (10 kDa) was successfully added to the RNaseB. The reaction mixture after the GaIT-1 reaction containing RNase B (6.7 µg, about 0.45 nmol) was dialyzed against H<sub>2</sub>O for 1 hour at room temperature and incubated at 37°C for 15.5 hours with 55 mU of the recombinant ST3Gal III in Tris-HCl buffer (50 mM, pH 7.3) containing CMP-SA-PEG (10 KDa) (0.25 mM) and MnCl<sub>2</sub> (20 mM) in a total volume of 20 µl. PEG-modified sialic acid residues were successfully added to the Gal-GlcNAc-

Man<sub>5</sub>GlcNAc<sub>2</sub>-peptide by the recombinant ST3Gal III. The yield can be further improved by adjusting the reaction conditions.

Conversion of high mannose N-Glycans to complex N-Glycans. To achieve this conversion, a GlcNAc $\beta$ 1,2Man<sub>3</sub>GlcNAc<sub>2</sub>-peptide intermediate is obtained. As shown in Figure 100, there are at least four feasible routes to carry out the reaction from Man<sub>5</sub>GlcNAc<sub>2</sub>-peptide to this intermediate:

**Route I:** The Man<sub>5</sub>GlcNAc<sub>2</sub>-peptide produced by the fungal  $\alpha$ 1,2 mannosidase is a substrate of GlcNAc transferase I (GlcNAcT-I, enzyme 2) which adds one GlcNAc. The terminal  $\alpha$ 1,3- and  $\alpha$ 1,6-linked mannose residues of GlcNAcMan<sub>5</sub>GlcNAc<sub>2</sub>-peptide is removed by Golgi  $\alpha$ -mannosidase II (ManII, enzyme 5). This route is a part of the natural pathway for the processing of N-linked oligosaccharides carried out in higher organisms.

**Route II:** Two mannose residues are first removed by an  $\alpha$ -mannosidase (enzyme 6), then a GlcNAc is added by GlcNAcT-I (enzyme 2). Other than its natural acceptor Man<sub>5</sub>GlcNAc<sub>2</sub>-R, GlcNAcT-I can also recognize Man<sub>3</sub>GlcNAc<sub>2</sub>-R as its substrate and add one GlcNAc to the mannose core structure to form GlcNAcMan<sub>3</sub>GlcNAc<sub>2</sub>-peptide.

**Route III:** The  $\alpha$ 1,6-linked mannose is removed by an  $\alpha$ 1,6-mannosidase, followed by the addition of GlcNAc by GlcNAcT-I and removal of the terminal  $\alpha$ 1,3-linked mannose by an  $\alpha$ 1,3-mannosidase. From the experimental data obtained, GlcNAcT-I can recognize this Man<sub>4</sub>GlcNAc<sub>2</sub>-peptide as acceptor and add one GlcNAc residue to form GlcNAcMan<sub>4</sub>GlcNAc<sub>2</sub>-peptide.

**Route IV:** Similar to Route III,  $\alpha$ 1,3-linked mannose is removed by an  $\alpha$ 1,3-mannosidase, followed by GlcNAcT-I reaction. Then the terminal  $\alpha$ 1,6-linked mannose can be removed by an  $\alpha$ 1,6-mannosidase.

After the function of GlcNAcT-I (responsible for the addition of the GlcNAc  $\beta$ 1,2-linked to the  $\alpha$ 1,3-mannose on the mannose core) and GlcNAcT-II (responsible for the addition of a second GlcNAc  $\beta$ 1,2-linked to the  $\alpha$ 1,6-mannose on the mannose core), the GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-peptide can be processed by GaIT 1 and sialyltransferase to form bi-antennary complex N- Glycans. Other GlcNAc transferases such as GlcNAcT-IV, GlcNAcT-V, and/or GlcNAcT-VI (Figure 100 and Figure 101) can also glycosylate the GlcNAc<sub>2</sub>Man<sub>3</sub>GlcNAc<sub>2</sub>-peptide. Additional glycosylation by the GaIT 1 and

sialyltransferases will form multi-antennary complex N-glycans. The enzyme GlcNAcT-III catalyzes the insertion of a bisecting GlcNAc, thus preventing the actions of ManII, GlcNAcT-II, GlcNAcT-IV and GlcNAcT-V.

**5      7. Preparation of EPO with multi-antennary complex glycans.**

This example sets forth the preparation of PEGylated, biantennary EPO, and triantennary, sialylated EPO from insect cell expressed EPO.

10     Recombinant human erythropoietin (rhEPO) from the baculovirus/Sf9 expression system (Protein Sciences Corp., Meriden, CT) was subjected to glycan analysis and the resulting glycans were shown to be primarily trimannosyl core with core fucose, with a small percentage of glycans also having a single GlcNAc (EPO 1).

15     **Addition of N-acetylglucosamine with GnT-I and GnT-II.** Two lots of rhEPO (1 mg/mL) were incubated with GnT-I and GnT-II, 5 mM UDP-glcNAc, 20 mM MnCl<sub>2</sub>, and 0.02% sodium azide in 100 mM MES pH 6.5 at 32°C for 24h. Lot A contained 20 mg of 20 EPO, and 100 mU/mL GnT-I and 60 mU/mL GnT-II. Lot B contained 41 mg of EPO, and 41 mU/mL GnT-I + 50 mU/mL GnT-II. After the reaction, the sample was desalted by gel filtration (PD10 columns, Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

20     **EPO glycans analyzed by 2-AA HPLC profiling.** This assay is a slight modification on Anumula and Dhume, Glycobiology 8 (1998) 685-69. Reductively-aminated N-glycans were injected onto a Shodex Asahipak NH2P-50 4D amino column (4.6 mm x 150 mm). Two solvents were used for the separation, A) 5% (v/v) acetic acid, 1% tetrahydrofuran, and 3% triethylamine in water and B) 2% acetic acid and 1% tetrahydrofuran in acetonitrile. The column was then eluted isocratically with 70% B for 2.5 min, followed by a linear gradient over a period of 100 min going from 70 to 5% B, and a final isocratic elution with 5% B for 20 min. Eluted peaks were detected using fluorescence detection with an excitation of 230 nm and emission wavelength of 420 nm. Non-sialylated N-linked glycans fall in the LC range of 23-34 min, monosialylated from 34-42 min, disialylated from 42-52 min, trisialylated from 55-65 min and tetrasialylated from 68 – 78 min.

25     Glycan profiling by 2AA HPLC revealed that lot A was 92% converted to a biantennary structure with two GlcNAcs (the balance having a single GlcNAc). Lot B showed 97% conversion to the desired product (Figure 102A and 102B ).

**Introducing a third antennary branch with GnT-V.** EPO (1 mg/mL of lot B) from the product of the GnT-I and GnT-II reactions, after desalting on PD-10 columns and subsequent concentration, was incubated with 10 mU/mL GnT-V and 5 mM UDP-GlcNAc in 100 mM MES pH 6.5 containing 5 mM MnCl<sub>2</sub> and 0.02% sodium azide at 32°C for 24 hrs.

- 5 2AA HPLC analysis demonstrated that the conversion occurred with 92% efficiency (Figure 103).

After desalting (PD-10) and concentration, galactose was added with rGalT1: EPO (1 mg/mL) was incubated with 0.1 U/mL GalT1, 5 mM UDP-galactose, 5 mM MnCl<sub>2</sub> at 32°C for 24 hrs.

- 10 **MALDI analysis of reductively-aminated N-glycans from EPO.** A small aliquot of the PNGase released N-glycans from EPO that had been reductively labeled with anthranilic acid was dialyzed for 45 min on an MF-Millipore membrane filter (0.025 µm pore, 47 mm dia), which was floating on water. The dialyzed aliquot was dried in a speedvac, redissolved in a small amount of water, and mixed with a solution of 2,5-dihydroxybenzoic acid (10 g/L) dissolved in water/acetonitrile (50:50). The mixture was dried onto the target and analyzed using an Applied Biosystems DE-Pro MALDI-TOF mass spectrometer operated in the linear/negative-ion mode. Oligosaccharides were assigned based on the observed mass-to-charge ratio and literature precedence.

- 15 Analysis of released glycans by MALDI showed that galactose was added quantitatively to all available sites (Figure 104). Galactosylated EPO from above was then purified by gel filtration on a Superdex 1.6/60 column in 50 mM Tris, 0.15M NaCl, pH 6.

- 20 **Sialylation.** After concentration and desalting (PD-10), 10 mg galactosylated EPO (1 mg/mL) was incubated with ST3Gal3 (0.05 U/mL), and CMP-SA (3 mM) in 50 mM Tris, 150 mM NaCl, pH 7.2 containing 0.02% sodium azide. A separate aliquot contained 25 radiolabelled CMP-SA. The resulting incorporated label and free label was separated by isocratic size exclusion chromatography/HPLC at 0.5 mL/min in 45% MeOH, 0.1%TFA (7.8mm x 30 cm column, particle size 5 µm, TSK G2000SW<sub>XL</sub>, Toso Haas, Ansys Technologies, Lake Forest, CA). Using this procedure, 12% of the counts were incorporated (360 micromolar, at 33 micromolar EPO, or about 10.9 moles/mole). Theoretical (3 N-linked sites, tri-antennary) is about 9 moles/mole incorporation. These correspond within the limits of the method. In an identical reaction with ST6Gal1 instead of ST3Gal3, 5.7% of the

radiolabel was incorporated into the galactosylated EPO, or about 48% compared with ST3Gal3.

B. GlycoPEGylation

5       8. Preparation of CMP-SA-PEG

This example sets forth the preparation of CMP-SA-PEG.

**Preparation of 2-(benzyloxycarboxamido)-glycylamide-2-deoxy-D-**

**mannopyranose.** N-benzyloxycarbonyl-glycyl-N-hydroxysuccinimide ester (3.125 g, 10.2 mmol) was added to a solution containing D-mannosamine-HCl (2 g, 9.3 mmol) and 10 triethylamine (1.42 mL, 10.2 mmol) dissolved in MeOH (10 mL) and H<sub>2</sub>O (6 mL). The reaction was stirred at room temperature for 16 hours and concentrated using rotoevaporation. Chromatography (silica, 10% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) yielded 1.71 g (50% yield) of product as a white solid: R<sub>f</sub> = 0.62 (silica; CHCl<sub>3</sub>:MeOH:H<sub>2</sub>O, 6/4/1); <sup>1</sup>H NMR (CD<sub>3</sub>OD, 500 MHz) δ 3.24-3.27 (m, 2H), 3.44 (t, 1H), 3.55 (t, 1H), 3.63-3.66 (m, 1H), 3.76-3.90 (m, 6H), 3.91 (s, 2H), 4.0 (dd, 2H), 4.28 (d, 1H, J = 4.4), 4.41 (d, 1H, J = 3.2), 5.03 (s, 1H), 5.10 (m, 3H), 7.29-7.38 (m, 10H).

**Preparation of 5-(N-benzyloxycarboxamido)glycylamide-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosuronate.** 2-(N-Benzylloxycarboxamido) glycylamide-2-deoxy-D-mannopyranose (1.59 g, 4.3 mmol) was dissolved in a solution of 0.1 M HEPES (12 mL, pH 7.5) and sodium pyruvate (4.73 g, 43 mmol). Neuraminic acid aldolase (540 U of enzyme in 45 mL of a 10 mM phosphate buffered solution containing 0.1 M NaCl at pH 6.9) and the reaction mixture was heated to 37 °C for 24 hr. The reaction mixture was then centrifuged and the supernatant was chromatographed (C18 silica, gradient from H<sub>2</sub>O (100%) to 30% MeOH/water). Appropriate fractions were pooled, concentrated and the residue chromatographed (silica, gradient from 10% MeOH/CH<sub>2</sub>Cl<sub>2</sub> to CH<sub>2</sub>Cl<sub>2</sub>/MeOH/H<sub>2</sub>O 6/4/1). 20 Appropriate fractions were collected, concentrated and the residue resuspended in water. After freeze-drying, the product (1.67 g, 87% yield) was obtained as a white solid: R<sub>f</sub> = 0.26 (silica, CHCl<sub>3</sub>/MeOH/H<sub>2</sub>O 6/4/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz) δ 1.82 (t, 1H), 2.20 (m, 1H), 3.49 (d, 1H), 3.59 (dd, 1H), 3.67-3.86 (m, 2H), 3.87 (s, 2H), 8.89-4.05 (m, 3H), 5.16 (s, 2H), 30 7.45 (m, 5H).

**Preparation of 5-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate.** 5-(N-Benzylloxycarboxamido)glycylamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosuronate (1.66 g, 3.6 mmol) was dissolved in 20 mL of 50% water/methanol. The flask was repeatedly evacuated and placed under argon and then 10% Pd/C (0.225 g) was added. After repeated evacuation, hydrogen (about 1 atm) was then added to the flask and the reaction mixture stirred for 18 hr. The reaction mixture was filtered through celite, concentrated by rotary evaporation and freeze-dried to yield 1.24 g (100% yield) of product as a white solid:  $R_f$  = 0.25 (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.83 (t, 1H, J = 9.9), 2.23 (dd, 1H, J = 12.9, 4.69), 3.51-3.70 (m, 2H), 3.61(s, 2H), 3.75-3.84 (m, 2H), 3.95-4.06(m, 3H).

**Preparation of cytidine-5'-monophosphoryl-[5-(N-fluorenylmethoxy-carboxamido)glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate].** A solution containing 5-glycylamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosuronate (0.55 g, 1.70 mmol) dissolved in 20 mL H<sub>2</sub>O was added to a solution 15 of Tris (1.38 g, 11.4 mmol), 1 M MgCl<sub>2</sub> (1.1 mL) and BSA (55 mg). The pH of the solution was adjusted to 8.8 with 1M NaOH (2 mL) and CTP-2Na<sup>+</sup> (2.23 g, 4.2 mmol) was added. The reaction mixture pH was controlled with a pH controller which delivered 1 M NaOH as needed to maintain pH 8.8. The fusion protein (sialyltransferase/CMP-neuraminic acid synthetase) was added to the solution and the reaction mixture was stirred at room 20 temperature. After 2 days, an additional amount of fusion protein was added and the reaction stirred an additional 40 hours. The reaction mixture was precipitated in EtOH and the precipitate was washed 5 times with cold EtOH to yield 2.3 grams of a white solid. About 1.0 g of the crude product was dissolved in 1,4 dioxane (4 mL), H<sub>2</sub>O (4 mL) and saturated NaHCO<sub>3</sub> (3 mL) and a solution of FMOC-Cl (308 mg, 1.2 mmol) dissolved in 2 ml dioxane 25 was added dropwise. After stirring for 16 h at room temperature, the reaction mixture was concentrated to about 6 mL by rotary evaporation and purified using chromatography (C18 silica, gradient 100% H<sub>2</sub>O to 30% MeOH/H<sub>2</sub>O). Appropriate fractions were combined and concentrated. The residue was dissolved in water and freeze-dried to yield 253 mg of a white solid:  $R_f$  = 0.50 (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.64 (dt, 1H, J = 12.0, 6.0), 2.50 (dd, 1H, J = 13.2, 4.9), 3.38 (d, J = 9.67, 1H), 3.60 (dd, J=11.65, 6.64, 1H), 3.79 (d, J=4.11, 1H), 3.87 (dd, J= 12.24, 1.0, 1H), 3.97 (m, 2H), 4.07 (td, J = 10.75, 4.84,

1H), 4.17 (dd, J = 10.68, 1.0, 1 H), 4.25 (s, 2H), 4.32 (t, J = 4.4, 1H), 4.37 (t, J = 5.8 1H), 4.6-4.7 (m, obscured by solvent peak), 5.95 (d, J = 4, 1 H), 6.03 (d, J = 7.4, 1H), 7.43-7.53 (m, 3H), 7.74 (m, 2H), 7.94 (q, J = 7, 3H). MS (ES); calc. for C<sub>35</sub>H<sub>42</sub>N<sub>5</sub>O<sub>18</sub>P ([M-H]<sup>-</sup>), 851.7; found 850.0.

5       **Preparation of cytidine-5'-monophosphoryl-(5-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate).** Diisopropylamine (83 uL, 0.587  $\mu$ mol) was added to a solution of cytidine-5'-monophosphoryl-[5-(N-fluorenyl-methoxycarboxamido)glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate] (100 mg, 0.117 mmol) dissolved in water (3 mL) and methanol (1 mL). The reaction mixture was stirred 16 h at room temperature and the reaction methanol removed from the reaction mixture by rotary evaporation. The crude reaction mixture was filtered through a C18 silica gel column using water and the eluant was collected and freeze-dried to yield (87 mg, 100%) of product as a white solid: R<sub>f</sub> = 0.21 (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.66 (td, 1H, J = 5.3), 2.50 (dd, 1H, J = 13.2, 4.6), 3.43 (d, J = 9.58, 1H), 3.63 (dd, J = 11.9, 6.44, 1H), 3.88 (dd, J = 11.8, 1.0, 1H), 3.95 (td, J = 9.0, 2.3, 1H), 4.10 (t, J = 10.42, 1H), 4.12 (td, J = 10.34, 4.66, 1 H), 4.18 (d, J = 10.36, 1H), 4.24 (m, 2H), 4.31 (t, J = 4.64, 1H), 4.35 (t, 1H), 6.00 (d, J = 4.37, 1 H), 6.13 (d, J = 7.71, 1H), 7.98 (d, J = 7.64, 1H). MS (ES); calc. for C<sub>21</sub>H<sub>32</sub>N<sub>5</sub>O<sub>11</sub>P ([M-H]<sup>-</sup>), 629.47; found 627.9.

10       **Preparation of cytidine-5'-monophosphoryl-[5-(N-methoxy-polyoxyethylene-(1 KDa)-3-oxypropionamido)-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate].** Benzyltriazol-1-yloxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP, 21 mg, 48  $\mu$ mol) was added to a solution of methoxypolyoxyethylene-(1 KDa average molecular weight)-3-oxypropionic acid (48 mg, 48  $\mu$ mol) dissolved in anhydrous DMF (700  $\mu$ L) and triethylamine (13  $\mu$ L, 95  $\mu$ mol). After 30 min, a solution containing cytidine-5'-monophosphoryl-(5-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate) (30 mg, 48  $\mu$ mol), water (400  $\mu$ L) and triethylamine (13  $\mu$ L, 95  $\mu$ mol) was added. This solution was stirred 20 min at room temperature and then chromatographed (C18 silica, gradient of methanol/water). Appropriate fractions were collected, concentrated, the residue dissolved in water and freeze-dried to afford 40 mg (50% yield) of a white solid: R<sub>f</sub> = 0.36 (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.66 (td, 1H, J = 5.3), 2.50 (dd, 1H, J = 13.2, 4.6), 2.64 (t, J = 5.99,

3H) 3.43 (d, J = 9.58, 1H), 3.63 (m, 1H), 3.71 (s, 70H), 3.79 (m, obscured by 3.71 peak), 3.82 (t, J=6.19, 1H) 3.88 (dd, J = 11.8, 1.0, 1H), 3.95 (td, J= 9.0, 2.3, 1H), 3.98 (t, J= 5.06, 1H), 4.12 (td, J = 10.34, 4.66, 1 H), 4.18 (d, J = 10.36, 1H), 4.23 (d, J=4.85, 2H), 4.31 (t, J=4.64, 1H), 4.35 (t, 1H), 6.00 (d, J = 4.55, 1 H), 6.13 (d, J = 7.56, 1H), 7.98 (d, J=7.54, 1H). MS (MALDI), observe [M- H]<sup>-</sup>; 1594.5, 1638.5, 1682.4, 1726.4, 1770.3, 1814.4, 1858.2, 1881.5, 1903.5, 1947.3.

Preparation of cytidine-5'-monophosphoryl-[5-(N-methoxy-polyoxyethylene-(10 KDa)-oxycarboxamido)-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate]. Cytidine-5'-monophosphoryl-(5-glycylamido-3,5-dideoxy- $\beta$ -D-glycero-D-galacto-2-nonulopyranosuronate) (2.5 mg, 4  $\mu$ mol) and water (180  $\mu$ L) was added to a solution of (Methoxypolyoxyethylene-(10 KDa, average molecular weight)-oxycarbonyl-(N-oxybenzotriazole) ester (40 mg, 4  $\mu$ mol) in anhydrous DMF (800  $\mu$ L) containing triethylamine (1.1  $\mu$ L, 8  $\mu$ mol) and the reaction mixture stirred for 1 hr at room temperature. The reaction mixture was then diluted with water (8 mL) and was purified by reversed phase flash chromatography (C18 silica, gradient of methanol/water). Appropriate fractions were combined, concentrated, the residue dissolved in water and freeze-dried yielding 20 mg (46% yield) of product as a white solid: R<sub>f</sub> = 0.35 (silica, IPA/H<sub>2</sub>O/NH<sub>4</sub>OH 7/2/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.66 (td, 1H), 2.50 (dd, 1H), 2.64 (t, 3H) 3.55-3.7 (m, obscured by 3.71 peak), 3.71 (s, 488H), 3.72-4.0 (m, obscured by 3.71 peak), 4.23 (m, 3H), 4.31 (t, 1H), 4.35 (t, 1H), 6.00 (d, J = 4.77, 1 H), 6.12 (d, J = 7.52, 1H), 7.98 (d, J=7.89, 1H). MS (MALDI), observe [M- CMP+Na]<sup>-</sup>; 10780.

#### 9. GlycoPEGylation of Human pituitary-derived FSH

This example illustrates the assembly of a conjugate of the invention. Follicle Stimulating Hormone (FSH) is desialylated and then conjugated with CMP-(sialic acid)-PEG.

**Desialylation of Follicle Stimulating Hormone.** Follicle Stimulating Hormone (FSH) (Human Pituitary, Calbiochem Cat No. 869001), 1 mg, was dissolved in 500  $\mu$ L 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub>. This solution, 375  $\mu$ L, was transferred to a small plastic tube and to it was added 263 mU Neuraminidase II (*Vibrio cholerae*). The reaction mixture was shaken gently for 15 hours at 32 °C. The reaction mixture was added to N-(*p*-aminophenyl)oxamic acid-agarose conjugate, 600  $\mu$ L, pre-equilibrated with 50 mM

Tris-HCl pH 7.4, 150 mM NaCl and 0.05% NaN<sub>3</sub> and gently rotated 6.5 hours at 4 °C. The suspension was centrifuged for 2 minutes at 14,000 rpm and the supernatant was collected. The beads were washed 5 times with 0.5 mL of the buffer and all supernatants were pooled. The enzyme solution was dialyzed (7000 MWCO) for 15 hours at 4 °C with 2 L of a solution containing 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>, and then twice for 4 hours at 4 °C into 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The solution was concentrated to 2 µg/µL by Speed Vac and stored at -20 °C. Reaction samples were analyzed by IEF gels (pH 3-7) (Invitrogen) (Figure 105).

**Preparation of human pituitary-derived SA-FSH and PEG-SA-Follicle**

10 **Stimulating Hormone.** Desialylated FSH (100 µg, 50 µL) and CMP-sialic acid or CMP-SA-PEG (1kDa or 10kDa) (0.05 umol) were dissolved in 13.5 µL H<sub>2</sub>O (adjusted to pH 8 with NaOH) in 0.5 mL plastic tubes. The tubes were vortexed briefly and 40 mU ST3Gal3 (36.5 µL) was added (total volume 100 µL). The tubes were vortexed again and shaken gently for 24 hours at 32 °C. The reactions were stopped by freezing at -80 °C. Reaction samples of 15 µg were analyzed by SDS-PAGE (Figure 106), IEF gels (Figure 107) and MALDI-TOF.

15 Native FSH was also analyzed by SDS-PAGE (Figure 108)

20 **Analysis of SDS PAGE and IEF Gels of Reaction Products.** Novex Tris-Glycine 8-16% 1 mm gels for SDS PAGE analysis were purchased from Invitrogen. 7.5 µL (15 µg) of FSH reaction samples were diluted with 5 µL of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NaN<sub>3</sub> buffer, mixed with 15 µL sample loading buffer and 1 µL 9 M µ-

mercaptoproethanol and heated for 6 minutes at 85 °C. Gels were run as directed by Invitrogen and stained with Colloidal Blue Stain (Invitrogen).

25 FSH samples (15 µg) were diluted with 5 µL Tris buffer and mixed with 15 µL sample loading buffer (Figure 105). The samples were then applied to Isoelectric Focusing Gels (pH 3-7) (Invitrogen) (Figure 108). Gels were run and fixed as directed by Invitrogen and then stained with Colloidal Blue Stain.

10. GlycoPEGylation of recombinant FSH produced recombinantly in CHO cells

30 This example illustrates the assembly of a conjugate of the invention. Disialylated FSH was conjugated with CMP-(sialic acid)-PEG.

**Preparation of recombinant Asialo-Follicle Stimulation Hormone.** Recombinant Follicle Stimulation Hormone (rFSH) produced from CHO was used in these studies. The 7,500 IU of Gonal-F was dissolved in 8 mL of water. The FSH solution was dialyzed in 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub> and concentrated to 500 μL in a Centricon 5 Plus 20 centrifugal filter. A portion of this solution (400 μL) (~ 0.8 mg FSH) was transferred to a small plastic tube and to it was added 275 mU Neuraminidase II (*Vibrio cholerae*). The reaction mixture was mixed for 16 hours at 32 °C. The reaction mixture was added to prewashed N-(*p*-aminophenyl)oxamic acid-agarose conjugate (800 μL) and gently rotated for 24 hours at 4 °C. The mixture was centrifuged at 10,000 rpm and the supernatant was 10 collected. The beads were washed 3 times with 0.6 mL Tris-EDTA buffer, once with 0.4 mL Tris-EDTA buffer and once with 0.2 mL of the Tris-EDTA buffer and all supernatants were pooled. The supernatant was dialyzed at 4 °C against 2 L of 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution was then concentrated to 420 μL in a Centricon Plus 20 15 centrifugal filter and stored at -20 °C.

Native and desialylated rFSH samples were analyzed by SDS-PAGE and IEF (Figure 109). Novex Tris-Glycine 8-16% 1 mm gels were purchased from Invitrogen. Samples (7.5 μL, 15 μg) samples were diluted with 5 μL of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NaN<sub>3</sub> buffer, mixed with 15 μL sample loading buffer and 1 μL 9 M β-20 mercaptoethanol and heated for 6 minutes at 85 °C. Gels were run as directed by Invitrogen and stained with Colloidal Blue Stain (Invitrogen). Isoelectric Focusing Gels (pH 3-7) were purchased from Invitrogen. Samples (7.5 μL, 15 μg) were diluted with 5 μL Tris buffer and mixed with 15 μL sample loading buffer. Gels were loaded, run and fixed as directed by Invitrogen. Gels were stained with Colloidal Blue Stain. Samples of native and desialylated 25 FSH were also dialyzed against water and analyzed by MALDI-TOF.

**Sialyl-PEGylation of recombinant Follicle Stimulation Hormone.** Desialylated FSH (100 μg, 54 μL) and CMP-SA-PEG (1 kDa or 10kDa) (0.05 μmol) were dissolved in 28 μL 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2 in 0.5 mL plastic tubes. The tubes were vortexed briefly and 20 mU of ST3Gal3 was added (total volume 100 μL). The tubes 30 were vortexed again, mixed gently for 24 hours at 32 °C and the reactions stopped by freezing

at -80 °C. Samples of this reaction were analyzed as described above by SDS-PAGE gels (Figure 110), IEF gels (Figure 111) and MALDI-TOF MS.

MALDI was also performed on the PEGylated rFSH. During ionization, SA-PEG is eliminated from the N-glycan structure of the glycoprotein. Native FSH gave a peak at 13928; AS-rFSH (13282); resialylate r-FSH (13332); PEG100-rFSH (13515; 14960 (1); 16455 (2); 17796 (3); 19321 (4)); and PEG 10000 (23560 (1); 24790 (2); 45670 (3); and 56760 (4)).

#### 11. Pharmacokinetic Study of GlycoPEGylated FSH

This example sets forth the testing of the pharmacokinetic properties glycoPEGylated Follicle Stimulating Hormone (FSH) prepared according to the methods of the invention as compared to non-PEGylated FSH.

FSH, FSH-SA-PEG (1KDa) and FSH-SA-PEG (10 KDa) were radioiodinated using standard conditions (Amersham Biosciences, Arlington Heights, IL) and formulated in phosphate buffered saline containing 0.1% BSA. After dilution in phosphate buffer to the appropriate concentration, each of the test FSH proteins (0.4 µg, each) was injected intraverneously into female Sprague Dawley rats (250-300 g body weight) and blood drawn at time points from 0 to 80 hours. Radioactivity in blood samples was analyzed using a gamma counter and the pharmacokinetics analyzed using standard methods (Figure 112). FSH was cleared from the blood much more quickly than FSH-PEG(1KDa), which in turn was clear somewhat more quickly than FSH-PEG(10KDa).

#### 12. Bioassay for FSH peptides

This example sets forth a bioassay for follicle stimulating hormone (FSH) activity based on cultured Sertoli cells. This assay is useful to determine the bioactivity of FSH after glycan remodeling, including glycoconjugation.

This bioassay is based on the dose-response relationship that exists between the amount of estradiol produced when FSH, but not lutenizing hormone (LH), is added to cultured Sertoli cells obtained from immature old rats. Exogenous testosterone is converted to 17 $\beta$ -estradiol in the presence of FSH.

Seven to 10 days old Sprague-Dawley rats were used to obtain Sertoli cells. After sacrifice, testes were decapsulated and tissue was dispersed by incubation in collagenase (1 mg/ml), trypsin (1mg/ml), hyaluronidase (1 mg/ml) and DNases (5  $\mu$ g/ml) for 5 to 10 min. The tubule fragments settled to the bottom of the flask and were washed in PBS (1x). The  
5 tubule fragments were reincubated for 20 min with a media containing the same enzymes: collagenase (1 mg/ml), trypsin (1mg/ml), hyaluronidase (1 mg/ml) and DNases (5  $\mu$ g/ml).

The tubule fragments were homogenized and plated into a 24 well plate in a serum free media.  $5 \times 10^5$  cells were dispersed per well. After 48h incubation at 37° C and 5% CO<sub>2</sub>, fresh media was added to the cells. Composition of the serum free media: DMEM (1  
10 vol), Ham's F10 nutrient mixture (1 vol), insulin 1  $\mu$ g/ml, Transferrin 5  $\mu$ g/ml, EGF 10 ng/ml, T4 20 pg/ml, Hydrocortisone 10<sup>-8</sup> M, Retinoic acid 10<sup>-6</sup> M.

The stimulation experiment consists of a 24 hour incubation with standard FSH or samples at 37°C and 5% CO<sub>2</sub>. The mean intra-assay coefficient of variation is 9% and the mean inter-assay coefficient of variation is 11%.

15 The 17B-estradiol Elisa Kit DE2000 (R&D Systems, Minneapolis, MN) was used to quantify the level of estradiol after incubation with FSH, FSH-SA-PEG (1KD<sub>a</sub>) and FSH-SA-PEG (10KD<sub>a</sub>).

The procedure was as follows: 100  $\mu$ l of Estradiol Standard (provided with kit and prepared as per instructions with kit) or sample was pipetted into wells of 17B-estradiol Elisa  
20 plate(s); 50  $\mu$ l of 17B-estradiol Conjugate (provided with kit, prepared as per instructions with kit) was added to each well; 50  $\mu$ l of 17B-estradiol Antibody solution (provided with kit and prepared as per instructions with kit) was added to each well; plates were incubated for 2 hour at room temperature at 200 rpm; the liquid was aspirated from each well; the wells were washed 4 times using the washing solution; all the liquid was removed from the wells; 200  $\mu$ l of pNPP Substrate (provided with kit and prepared as per instructions with kit) was added to all wells and incubated for 45 min; 50  $\mu$ l of Stop solution (provided with kit and prepared as per instructions with kit) was added and the plates were read it at 405 nm (Figure 113). While FSH-PEG(10KD<sub>a</sub>) exhibited a modest stimulation of Sertoli cells, at 1  $\mu$ g/ml, FSH-PEG(1KD<sub>a</sub>) stimulated Sertoli cells up to 50% more than unPEGylated FSH.

### 13. GlycoPEGylation of Transferrin

This example sets forth the preparation of asialotransferrin and its sialylation with PEG-CMP-sialic acid.

**Preparation of Asialo-transferrin.** Human-derived holo-Transferrin, (10 mg) was dissolved in 500  $\mu$ L of 50 mM NaOAc, 5 mM CaCl<sub>2</sub>, pH 5.5. To this solution was added 500 mU Neuraminidase II (*Vibrio cholerae*) and the reaction mixture was shaken gently for 20.5 hours at 37 °C. The reaction mixture was added to the prewashed N-(*p*-aminophenyl)oxamic acid-agarose conjugate (600  $\mu$ L) and the washed beads gently rotated for 24 hours at 4 °C. The mixture was centrifuged at 10,000 rpm and the supernatant was 10 collected. The reaction mixture was adjusted to 5 mM EDTA by addition of 100  $\mu$ L of 30 mM EDTA to the washed beads, which were gently rotated for 20 hours at 4 °C. The suspension was centrifuged for 2 minutes at 10,000 rpm and the supernatant was collected. The beads were washed 5 times with 0.35 mL of 50 mM NaOAc, 5 mM CaCl<sub>2</sub>, 5 mM 15 EDTA, pH 5.5 and all supernatants were pooled. The enzyme solution was dialyzed twice at 4 °C into 15 mM Tris-HCl, 1 M NaCl, pH 7.4. 0.3 mL of the transferrin solution (3.3 mL total) was removed and dialyzed twice against water. The remainder was dialyzed twice more at 4 °C against phosphate buffered saline. The dialyzed solution was stored at -20 °C. Protein samples were analyzed by IEF Electrophoresis. Samples (9  $\mu$ L, 25  $\mu$ g) were diluted with 16  $\mu$ L Tris buffer and mixed with 25  $\mu$ L of the sample loading buffer and applied to 20 Isoelectric Focusing Gels (pH 3-7). Gels were run and fixed using standard procedures. Gels were stained with Colloidal Blue Stain.

**Sialyl-PEGylation of asialo-Transferrin.** Desialylated transferrin (250  $\mu$ g) and CMP-sialic acid or CMP-SA-PEG (1kDa or 10kDa)(0.05  $\mu$ mol) were dissolved in 69  $\mu$ L 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2 in 1.5 mL plastic tubes. The tubes were 25 vortexed briefly and 100 mU ST3Gal3 (90  $\mu$ L) were added (total volume 250  $\mu$ L). The tubes were vortexed again and mixed gently for 24 hours at 32 °C. The reactions were stopped by freezing at -80 °C. Novex Tris-Glycine 8-16% 1 mm gels were used for SDS PAGE analysis (Figure 114). Samples (25  $\mu$ L, 25  $\mu$ g) were mixed with 25  $\mu$ L of sample loading buffer and 0.4  $\mu$ L of  $\beta$ -mercaptoethanol and heated for 6 minutes at 85 °C. Gels 30 were run using standard conditions and stained with Colloidal Blue Stain. IEF gels were also

performed as described above Figure 115). Samples were also dialyzed against water analyzed by MALDI-TOF.

**Results.** MALDI was also performed. Native transferrin (78729); asialotransferrin (78197); resialylated transferrin (79626/80703); with SA-PEG 1k (79037 (1); 80961 (2); 5 82535 (3); 84778 (4)); with SA-PEG 5k (90003 (2); 96117 (3); 96117 (4)); with SA-PEG 10k (100336 (2); 111421 (3); 122510 (4)).

#### 14. GlycoPEGylation of Recombinant Factor VIIa produced in BHK cells

This example sets forth the PEGylation of recombinant Factor VIIa made in CHO

10 cells.

**Preparation of Asialo-Factor VIIa.** Recombinant Factor VIIa was produced in BHK cells (baby hamster kidney cells). Factor VIIa (14.2 mg) was dissolved at 1 mg/ml in buffer solution (pH 7.4, 0.05 M Tris, 0.15 M NaCl, 0.001 M CaCl<sub>2</sub>, 0.05% NaN<sub>3</sub>) and was incubated with 300 mU/mL sialidase (*Vibrio cholera*)-agarose conjugate for 3 days at 32 °C.

15 To monitor the reaction a small aliquot of the reaction was diluted with the appropriate buffer and an IEF gel performed according to Invitrogen procedures (Figure 116). The mixture was centrifuged at 3,500 rpm and the supernatant was collected. The resin was washed three times (3×2 mL) with the above buffer solution (pH 7.4, 0.05 M Tris, 0.15 M NaCl, 0.05% NaN<sub>3</sub>) and the combined washes were concentrated in a Centricon-Plus-20. The remaining 20 solution was buffer exchanged with 0.05 M Tris (pH 7.4), 0.15 M NaCl, 0.05% NaN<sub>3</sub> to a final volume of 14.4 mL.

25 **Preparation of Factor VIIa-SA-PEG (1 KDa and 10 KDa).** The desialylation rFactor VIIa solution was split into two equal 7.2 ml samples. To each sample was added either CMP-SA-5-PEG(1KDa) (7.4 mg) or CMP-SA-5-PEG(10KDa) (7.4 mg). ST3Gal3 (1.58U) was added to both tubes and the reaction mixtures were incubated at 32°C for 96 hrs. The reaction was monitored by SDS-PAGE gel using reagents and conditions described by Invitrogen. When the reaction was complete, the reaction mixture was purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The combined fractions containing the product were concentrated 30 at 4°C in Centricon-Plus-20 centrifugal filters (Millipore, Bedford, MA) and the concentrated solution reformulated to yield 1.97 mg (bicinchoninic acid protein assay, BCA assay, Sigma-

Aldrich, St. Louis MO) of Factor VIIa-PEG. The product of the reaction was analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples were dialyzed against water and analyzed by MALDI-TOF. Figure 117 shows the MALDI results for native Factor VIIa. Figure 118 contains the MALDI results for 5 Factor VIIa PEGylated with 1kDa PEG where peak of Factor VIIa PEGylated with 1kDa PEG is evident. Figure 119 contains the MALDI results for Factor VIIa PEGylated with 10kDa PEG where a peak for Factor VIIa PEGylated with 10kDa PEG is evident. Figure 120 depicts the SDS-PAGE analysis of all of the reaction products, where a band for Factor VIII-SA-PEG(10-kDa) is evident.

10

#### 15. GlycoPEGylation of Factor IX produced in CHO cells

This example sets forth the preparation of asialoFactor IX and its sialylation with PEG-CMP-sialic acid.

Desialylation of rFactor IX. A recombinant form of Coagulation Factor IX (rFactor 15 IX) was made in CHO cells. 6000 IU of rFactor IX were dissolved in a total of 12 mL USP H<sub>2</sub>O. This solution was transferred to a Centricon Plus 20, PL-10 centrifugal filter with another 6 mL USP H<sub>2</sub>O. The solution was concentrated to 2 mL and then diluted with 15 mL 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub>, 0.05% NaN<sub>3</sub> and then reconcentrated. The dilution/concentration was repeated 4 times to effectively change the buffer to a final 20 volume of 3.0 mL. Of this solution, 2.9 mL (about 29 mg of rFactor IX) was transferred to a small plastic tube and to it was added 530 mU α2-3,6,8-Neuraminidase- agarose conjugate (*Vibrio cholerae*, Calbiochem, 450 μL). The reaction mixture was rotated gently for 26.5 hours at 32 °C. The mixture was centrifuged 2 minutes at 10,000 rpm and the supernatant was collected. The agarose beads (containing neuraminidase) were washed 6 times with 0.5 25 mL 50 mM Tris-HCl pH 7.12, 1 M NaCl, 0.05% NaN<sub>3</sub>. The pooled washings and supernatants were centrifuged again for 2 minutes at 10,000 rpm to remove any residual agarose resin. The pooled, desialylated protein solution was diluted to 19 mL with the same buffer and concentrated down to ~ 2 mL in a Centricon Plus 20 PL-10 centrifugal filter. The solution was twice diluted with 15 mL of 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% 30 NaN<sub>3</sub> and reconcentrated to 2 mL. The final desialylated rFactor IX solution was diluted to 3 mL final volume (~10 mg/mL) with the Tris Buffer. Native and desialylated rFactor IX

samples were analyzed by IEF-Electrophoresis. Isoelectric Focusing Gels (pH 3-7) were run using 1.5  $\mu$ L (15  $\mu$ g) samples first diluted with 10  $\mu$ L Tris buffer and mixed with 12  $\mu$ L sample loading buffer. Gels were loaded, run and fixed using standard procedures. Gels were stained with Colloidal Blue Stain (Figure 121), showing a band for desialylated Factor  
5 IX.

Preparation of PEG (1 kDa and 10 kDa)-SA-Factor IX. Desialylated rFactor-IX (29 mg, 3 mL) was divided into two 1.5 mL (14.5 mg) samples in two 15 mL centrifuge tubes. Each solution was diluted with 12.67 mL 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 0.05% NaN<sub>3</sub> and either CMP-SA-PEG-1k or 10k (7.25  $\mu$ mol) was added. The tubes were  
10 inverted gently to mix and 2.9 U ST3Gal3 (326  $\mu$ L) was added (total volume 14.5 mL). The tubes were inverted again and rotated gently for 65 hours at 32 °C. The reactions were stopped by freezing at -20 °C. 10  $\mu$ g samples of the reactions were analyzed by SDS-PAGE. The PEGylated proteins were purified on a Toso Haas Biosep G3000SW (21.5 x 30 cm, 13 um) HPLC column with Dulbecco's Phosphate Buffered Saline, pH 7.1 (Gibco), 6 mL/min.  
15 The reaction and purification were monitored using SDS Page and IEF gels. Novex Tris-Glycine 4-20% 1 mm gels were loaded with 10  $\mu$ L (10  $\mu$ g) of samples after dilution with 2  $\mu$ L of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% NaN<sub>3</sub> buffer and mixing with 12  $\mu$ L sample loading buffer and 1  $\mu$ L 0.5 M DTT and heated for 6 minutes at 85 °C. Gels were stained with Colloidal Blue Stain (Figure 122) showing a band for PEG (1 kDa and 10 kDa)-  
20 SA-Factor IX.

#### 16. Direct Sialyl-GlycoPEGylation of Factor-IX

This example sets forth the preparation of sialyl-glycoPEGylation of peptides without prior sialidase treatment. Here, Factor-IX is the exemplary peptide.

25 **Direct Sialyl-PEGylation (10 KDa) of Factor-IX.** Factor IX (1100 IU) was dissolved in 5 mL of 20 mM histidine, 520 mM glycine buffer containing 2% sucrose, 0.05% NaN<sub>3</sub>, and 0.01% polysorbate 80, pH 5.0. The CMP-SA-PEG (10 KDa) (27.8 mg, 3.5  $\mu$ mol) was then added to this solution, the reaction mixture inverted gently to mix and 1.4 U of ST3Gal3 was added. The reaction mixture was rotated gently for 19 hours at 32 °C and the  
30 reaction was stopped by freezing. The reaction mixture was analyzed by SDS-PAGE gels using development and staining (Colloidal Blue) conditions described by Invitrogen. Briefly,

samples (10  $\mu$ L) were mixed with 12  $\mu$ L sample loading buffer and 2  $\mu$ L 0.5 M DTT and heated for 6 minutes at 85 °C (Figure 123, lanes 8, 9 and 10). The product was purified on a Superdex 200 10/20 column (Amersham, Uppsala, Sweden) column with Dulbecco's Phosphate Buffered Saline, pH 7.1 (Gibco), 6 mL/min. Figure (Figure 123 contains a band 5 (lane 5) of the HPLC-purified PEGylated Factor-IX.

#### 17. Sialic Acid Capping of GlycoPEGylated Factor IX

This example sets forth the procedure for sialic acid capping of sialyl glycoPEGylated peptides. Here, Factor-IX is the exemplary peptide.

10 **Sialic acid capping of N-linked and O-linked Glycans of Factor-IX-SA-PEG (10KDa).** Purified r-Factor-IX-PEG (10 KDa) (2.4 mg) was concentrated in a Centricon® Plus 20 PL-10 (Millipore Corp., Bedford, MA) centrifugal filter and the buffer was changed to 50 mM Tris-HCl pH 7.2, 0.15 M NaCl, 0.05% NaN<sub>3</sub> to a final volume of 1.85 mL. The protein solution was diluted with 372  $\mu$ L of the same Tris buffer and 7.4 mg CMP-SA (12 15  $\mu$ mol) was added as a solid. The solution was inverted gently to mix and 0.1 U ST3Gal1 and 0.1 U ST3Gal3 were added. The reaction mixture was rotated gently for 42 hours at 32 °C.

A 10  $\mu$ g sample of the reaction was analyzed by SDS-PAGE. Novex Tris-Glycine 4-12% 1 mm gels were performed and stained using Colloidal Blue as described by Invitrogen. Briefly, samples, 10  $\mu$ L (10  $\mu$ g), were mixed with 12  $\mu$ L sample loading buffer and 1  $\mu$ L 0.5 20 M DTT and heated for 6 minutes at 85 °C (Figure 123, lane 4).

#### 18. GlycoPEGylation of Proteins expressed in Mammalian or Insect Systems: EPO, Interferon $\alpha$ and Interferon $\beta$

This example sets forth the preparation of PEGylated peptides that are expressed in 25 mammalian and insect systems.

**Preparation of acceptor from mammalian expression systems.** The peptides to be glycoPEGylated using CMP-sialic acid PEG need to have glycans terminating in galactose. Most peptides from mammalian expression systems will have terminal sialic acid that first needs to be removed.

30 **Sialidase digestion.** The peptide is desialylated using a sialidase. A typical procedure involves incubating a 1 mg/mL solution of the peptide in Tris-buffered saline, pH

7.2, with 5 mM CaCl<sub>2</sub> added, with 0.2 U/mL immobilized sialidase from *Vibrio cholera* (Calbiochem) at 32°C for 24 hours. Microbial growth can be halted either by sterile filtration or the inclusion of 0.02% sodium azide. The resin is then removed by centrifugation or filtration, and then washed to recover entrapped peptide. At this point, EDTA may be added 5 to the solution to inhibit any sialidase that has leached from the resin.

**Preparation from insect expression systems.** EPO, interferon-alpha, and interferon-beta may also be expressed in non-mammalian systems such as yeast, plants, or insect cells. The peptides to be glycoPEGylated using CMP-sialic acid PEG need to have glycans terminating in galactose. The majority of the N-glycans on peptides expressed in 10 insect cells, for example, are the trimannosyl core. These glycans are first built out to glycans terminating in galactose before they are acceptors for sialyltransferase.

**Building acceptor glycans from trimannosyl core.** Peptide (1 mg/mL) in Tris-buffered saline, pH 7.2, containing 5 mM MnCl<sub>2</sub>, 5 mM UDP-GlcNAc, 0.05 U/mL GLCNACT I, 0.05 U/mL GLCNACT II, is incubated at 32°C for 24 hours or until the 15 reaction is substantially complete. Microbial growth can be halted either by sterile filtration or the inclusion of 0.02% sodium azide. After buffer exchange to remove UDP and other small molecules, UDP-galactose and MnCl<sub>2</sub> are each added to 5 mM, galactosyltransferase is added to 0.05 U/mL, and is incubated at 32°C for 24H or until the reaction is substantially complete. Microbial growth can be halted either by sterile filtration or the inclusion of 0.02% 20 sodium azide. The peptides are then ready for glycoPEGylation.

**Building O-linked glycans.** A similar strategy may be employed for interferon alpha to produce enzymatically the desired O-glycan Gal-GalNAc. If necessary, GalNAc linked to serine or threonine can be added to the peptide using appropriate peptide GalNAc transferases (e.g. GalNAc T1, GalNAc T2, T3, T4, etc.) and UDP-GalNAc. Also, if needed, 25 galactose can be added using galactosyltransferase and UDP-galactose.

**GlycoPEGylation using sialyltransferase.** The glycopeptides (1 mg/mL) bearing terminal galactose in Tris buffered saline + 0.02% sodium azide are incubated with CMP-SA-PEG (0.75 mM) and 0.4 U/mL sialyltransferase (ST3Gal3 or ST3Gal4 for N-glycans on EPO and interferon beta; ST3Gal4, or ST3Gal1 for O-glycans on interferon alpha) at 32°C for 24 30 hours. Other transferases that may work include the 2,6 sialyltransferase from *Photobacterium damsella*. The acceptor peptide concentration is most preferably in the range

of 0.1 mg/mL up to the solubility limit of the peptide. The concentration of CMP-SA-PEG should be sufficient for there to be excess over the available sites, but not so high as to cause peptide solubility problems due to the PEG, and may range from 50  $\mu$ M up to 5 mM, and the temperature may range from 2°C up to 40°C. The time required for complete reaction will  
5 depend on the temperature, the relative amounts of enzyme to acceptor substrate, the donor substrate concentration, and the pH.

#### 19. GlycoPEGylation of EPO produced in insect cells

This example sets forth the preparation of PEGylated biantennary EPO from insect cell  
10 expressed EPO.

Recombinant human erythropoietin (rhEPO) from the baculovirus/Sf9 expression system (Protein Sciences Corp., Meriden, CT) was subjected to glycan analysis and the resulting glycans were shown to be primarily trimannosyl core with core fucose, with a small percentage of glycans also having a single GlcNAc (Figure 124).

15       **Addition of N-acetylglucosamine with GnT-I and GnT-II.** Two lots of rhEPO (1 mg/mL) were incubated with GnT-I and GnT-II, 5 mM UDP-GlcNAc, 20 mM MnCl<sub>2</sub>, and 0.02% sodium azide in 100 mM MES pH 6.5 at 32°C for 24h. Lot A contained 20 mg of EPO, and 100 mU/mL GnT-I and 60 mU/mL GnT-II. Lot B contained 41 mg of EPO, and 41 mU/mL GnT-I + 50 mU/mL GnT-II. After the reaction, the sample was desalted by gel  
20 filtration (PD10 columns, Pharmacia LKB Biotechnology Inc., Piscataway, NJ).

Glycan profiling by 2AA HPLC revealed that lot A was 92% converted to a biantennary structure with two GlcNAcs (the balance having a single GlcNAc. Lot B showed 97% conversion to the desired product (Figure 102A and 102B ).

25       **Galactosylation of EPO lot A.** EPO (~16 mgs of lot A) was treated with GnTII to complete the addition of GlcNAc. The reaction was carried out in 50 mM Tris pH 7.2 containing 150 mM NaCl, EPO mg/ml, 1 mM UDP-GlcNAc, 5 mM MnCl<sub>2</sub>, 0.02% sodium azide and 0.02 U/ml GnTII at 32 C for 4 hrs. Then galactosylation of EPO was done by adding UDP-galactose to 3 mM and GalT1 to 0.5 U/ml and the incubation continued at 32 C for 48 hrs.

30       Galactosylated EPO was then purified by gel filtration on a Superdex 1.6/60 column in 50 mM Tris, 0.15M NaCl, pH 6. The EPO containing peak was then analyzed by 2AA

HPLC. Based on the HPLC data ~85% of the glycans contains two galactose and ~15% of the glycans did not have any galactose after galactosylation reaction.

**Sialylation of galactosylated EPO.** Sialylation of galactosylated EPO was carried out in 100 mM Tris pH containing 150 mM NaCl, 0.5 mg/ml EPO, 200 mU/ml of ST3Gal3 and either 0.5 mM CMP-NAN or CMP-NAN-PEG (1 KDa) or CMP-NAN-PBG (10 KDa) for 5 48 hrs at 32 °C. Almost all of the glycans that have two galactose residues were fully sialylated (2 sialic acids / glycan) after sialylation reaction with CMP-NAN. MALDI-TOF analysis confirmed the HPLC data.

**PEGylation of galactosylated EPO.** For PEGylation reactions using CMP-NAN-10 PEG (1 KDa) and CMP-NAN-PEG (10 KDa), an aliquot of the reaction mixture was analyzed by SDS-PAGE (Figure 125). The molecular weight of the EPO peptide increased with the addition of each sugar, and increased more dramatically in molecular weight after the PEGylation reactions.

**In vitro bioassay of EPO.** *In vitro* EPO bioassay (adapted from Hammerling et al, 15 1996, J. Pharm. Biomed. Anal. 14: 1455-1469) is based on the responsiveness of the TF-1 cell line to multiple levels of EPO. TF-1 cells provide a good system for investigating the proliferation and differentiation of myeloid progenitor cells. This cell line was established by T. Kitamura et al. in October 1987 from a heparinized bone marrow aspiration sample from a 35 year old Japanese male with severe pancytopenia. These cells are completely dependent 20 on Interleukin 3 or Granulocyte-macrophage colony-stimulating factor (GM-CSF).

The TF-1 cell line (ATCC, Cat. No. CRL-2003) was grown in RPMI + FBS 10% + GM-CSF (12 ng/ml) and incubated at 37 °C 5% CO<sub>2</sub>. The cells were in suspension at a concentration of 5000 cells/ml of media, and 200 µl were dispensed in a 96 well plate. The cells were incubated with various concentrations of EPO (0.1 µg/ml to 10 µg/ml) for 48 hours. 25 A MTT Viability Assay was then done by adding 25 µl of MTT at 5 mg/ml (SIGMA M5655), incubating the plate at 37°C for 20 min to 4 hours, adding 100 µl of isopropanol/HCl solution (100 ml isopropanol + 333 µl HCl 6N), reading the OD at 570 nm, and 630nm or 690nm, and subtracting the readings at 630 nm or 690 nm from the readings at 570 nm.

30 Figure 126 contains the results when sialylated EPO, and EPO glycoPEGylated with 1 KDa or 10 KDa PEG was subjected to an *in vitro* EPO bioactivity test. The EPO

glycoPEGylated with 1KDa PEG had almost the same activity as the unglycoPEGylated EPO when both were at a concentration of approximately 5 µg/ml. The EPO glycoPEGylated with 10KDa PEG had approximately half the activity of the unglycoPEGylated EPO when both were at a concentration of approximately 5 µg/ml.

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#### 20. GlycoPEGylation of Interferon $\alpha$ produced in CHO cells

**Preparation of Asialo-Interferon  $\alpha$ .** Interferon alpha produced from CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub> and concentrated to 500 µL in a Centricon Plus 20 centrifugal filter. The solution is 10 incubated with 300 mU/mL Neuraminidase II (*Vibrio cholerae*) for 16 hours at 32 °C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel performed. The reaction mixture is then added to prewashed N-(*p*-aminophenyl)oxamic acid-agarose conjugate (800 µL/mL reaction volume) and the washed beads gently rotated for 24 hours at 4 °C. The mixture is centrifuged at 10,000 rpm and the 15 supernatant was collected. The beads are washed 3 times with Tris-EDTA buffer, once with 0.4 mL Tris-EDTA buffer and once with 0.2 mL of the Tris-EDTA buffer and all supernatants were pooled. The supernatant is dialyzed at 4 °C against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated using a Centricon Plus 20 20 centrifugal filter and stored at -20 °C. The conditions for the IEF gel are run according to the procedures and reagents provided by Invitrogen. Samples of native and desialylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Interferon-alpha-(alpha2,3)-Sialyl-PEG.** Desialylated interferon-alpha is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. 25 The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of ST3Gal1 at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated 30 using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions

based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples of native and desialylated Interferon-alpha are dialyzed against water and analyzed by MALDI-TOF MS.

5       **Preparation of Interferon-alpha-(alpha2,8)-Sialyl-PEG.** Interferon-alpha produced in CHO, which contains an alpha2,3-sialylated O-linked glycan, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of CST-II at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction has CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on 10 UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples of native and PEGylated interferon-alpha are dialyzed against water and analyzed by MALDI-TOF MS.

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**Preparation of Interferon-alpha-(alpha2,6)-Sialyl-PEG.** Interferon-alpha, containing only O-linked GalNAc, was dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of ST6GalNAcI or II at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples of native and PEGylated interferon-alpha are 20 dialyzed against water and analyzed by MALDI-TOF MS.

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21. GlycoPEGylation of G-CSF produced in CHO cells

**Preparation of Asialo-Granulocyte-Colony Stimulation Factor (G-CSF).** G-CSF produced in CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 5 mM CaCl<sub>2</sub> and concentrated to 500 µL in a Centricon Plus 20 centrifugal filter. The solution is incubated with 300 mU/mL Neuraminidase II (*Vibrio cholerae*) for 16 hours at 32 °C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel performed. The reaction mixture is then added to prewashed N-(*p*-aminophenyl)oxamic acid-agarose conjugate (800 µL/mL reaction volume) and the washed beads gently rotated for 24 hours at 4 °C. The mixture is centrifuged at 10,000 rpm 10 and the supernatant was collected. The beads are washed 3 times with Tris-EDTA buffer, once with 0.4 mL Tris-EDTA buffer and once with 0.2 mL of the Tris-EDTA buffer and all supernatants are pooled. The supernatant is dialyzed at 4 °C against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated using a Centricon Plus 20 15 centrifugal filter and stored at -20 °C. The conditions for the IEF gel were run according to the procedures and reagents provided by Invitrogen. Samples of native and desialylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of G-CSF-(alpha2,3)-Sialyl-PEG.** Desialylated G-CSF was dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is 20 incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of ST3Gal1 at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line 25 fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples of native and PEGylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

30       **Preparation of G-CSF-(alpha2,8)-Sialyl-PEG.** G-CSF produced in CHO cells, which contains an alpha2,3-sialylated O-linked glycan, is dissolved at 2.5 mg/mL in 50 mM

Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of CST-II at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction has CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a  
5 Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and  
10 reagents supplied by Invitrogen. Samples of native and PEGylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of G-CSF-(alpha2,6)-Sialyl-PEG.** G-CSF, containing only O-linked GaINAc, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-PEG and 0.1 U/mL of ST6GalNAcI or  
15 II at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction has CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. After 2 days, the reaction mixture is purified using a  
20 Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples of native and PEGylated G-CSF are dialyzed against water and analyzed by MALDI-TOF MS.

25 22. GlycoPEGylation of O-Linked Glycans of EPO produced in CHO Cells

**Preparation of O-linked EPO-SA-PEG (10 kDa).** Asialo-EPO, originally produced in CHO cells, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 5 mM CMP-SA and 0.1 U/mL of ST3Gal3 at  
32°C for 2 days. To monitor the incorporation of sialic acid onto the N-linked glycans, a  
30 small aliquot of the reaction had CMP-SA-<sup>14</sup>C added; the peptide is separated by gel filtration

on a Toso Haas G2000SW analytical column using methanol, water and the product detected using a radiation detector. When the reaction is complete, the solution is concentrated using a Centricon-20 filter. The remaining solution is buffer exchanged with 0.05 M Tris (pH 7.2), 0.15 M NaCl, 0.05% NaN<sub>3</sub> to a final volume of 7.2 mL until the CMP-SA could no longer be detected. The reteinate is then resuspended in 0.05 M Tris (pH 7.2), 0.15 M NaCl, 0.05% NaN<sub>3</sub> at 2.5 mg/mL protein. The solution is incubated with 1 mM CMP-SA-PEG (10 KDa) and ST3Gal1, to glycosylate the O-linked site, at 32°C for 2 days. To monitor the incorporation of sialic acid-PEG, a small aliquot of the reaction is separated by gel filtration using a Toso Haas TSK-gel-3000 analytical column eluting with PBS pH 7.0 and analyzing by UV detection. When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-gel-3000 preparative column using PBS buffer (pH 7.0) collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

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### 23. GlycoPEGylation of an antibody

This example sets forth the procedures to PEGylate the O-linked glycans of an antibody molecule. Here, Enbrel™ is used as an example, however one of skill in the art will appreciate that this procedure can be used with many antibody molecules.

20

**Preparation of Enbrel™-SA-PEG (10 KDa).** Enbrel™ (TNF-receptor-IgG<sub>1</sub>-chimera), either with the O-linked glycans sialylated prior to PEGylation or not, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 5 mM UDP-galactose and 0.1 U/mL of galactosyltransferase at 32°C for 2 days to cap the Fc region glycans with galactose. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G2000SW analytical column in methanol and water. The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

25

When the reaction is complete, the solution is incubated with 1 mM CMP-sialic acid-linker-PEG (10 KDa) and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the

incorporation of sialic acid-linker-PEG, the peptide is separated by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The 5 fractions containing product are combined, concentrated, buffer exchanged and then freeze-dried. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

10 24. GlycoPEGylation of Remicade™ antibody

This example sets forth the procedure to glycoPEGylate a recombinant antibody molecule by introducing PEG molecules to the Fc region glycans. Here Remicade™, a TNF-R:IgG Fc region fusion protein, is the exemplary peptide.

15 **Preparation of Remicade™-Gal-PEG (10 KDa).** Remicade™ is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM UDP-galactose-PEG (10 KDa) and 0.1 U/mL of galactosyltransferase at 32°C for 2 days to introduce the PEG in the Fc region glycans. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel 20 filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based 25 on UV absorption. The fractions containing product are combined, concentrated, buffer exchanged and then freeze-dried. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

25. Generation and PEGylation of GlcNAc-ASN structures: TPA produced in Yeast

This example sets forth the preparation of PEGylated GlcNAc-Asn structures on a peptide such as TPA expressed in yeast.

5        Yeast expression is expected to result in a TPA which contains a single N-linked mannan-type structure. This recombinant glycoprotein is first treated with endoglycosidase H to generate GlcNAc structures on the asparagine (Asn) residues on the peptide.

The GlcNAc-Asn structures on the peptide/protein backbone are then be modified with galactose or galactose-PEG using UDP-galactose or UDP-galactose-6-PEG, 10 respectively, and a galactosyltransferase such as GalT1. In one case, the galactose-PEG is the terminal residue. In the second case, the galactose is further modified with SA-PEG using a CMP-SA-PEG donor and a sialyltransferase such as ST3GalIII. In another embodiment, the GlcNAc-Asn structures on the peptide/protein backbone may be galactosylated and sialylated as described above, and then further sialylated using CMP-SA-15 PEG and an  $\alpha$ 2,8-sialyltransferase such as the enzyme encoded by the *Campylobacter jejuni* cst-II gene.

26. Generation and PEGylation of GlcNAc-ASN Structures: GM-CSF produced in *Saccharomyces*

20        This example sets forth the preparation of Tissue-type Activator with PEGylated GlcNAc-Asn structures.

Recombinant GM-CSF expressed in yeast is expected to contain 2 N-linked and 2 O-linked glycans. The N-linked glycans should be of the branched mannan type. This recombinant glycoprotein is treated with an endoglycosidase from the group consisting of 25 endoglycosidase H, endoglycosidase-F1, endoglycosidase-F2, endoglycosidase-F3, endoglycosidase-M either alone or in combination with mannosidases I, II and III to generate GlcNAc nubs on the asparagine (Asn) residues on the peptide/protein backbone.

The GlcNAc-Asn structures on the peptide/protein backbone is then be modified with galactose or galactose-PEG using UDP-galactose or UDP-galactose-6-PEG, respectively, and 30 a galactosyltransferase such as GalT1. In one case the galactose-PEG is the terminal residue. In the second case the galactose is further modified with SA-PEG using a CMP-SA-PEG

donor and a sialyltransferase such as ST3GalIII. In another embodiment the GlcNAc-Asn structures on the peptide/protein backbone can be galactosylated and sialylated as described above, and then further sialylated using CMP-SA-PEG and an  $\alpha$ 2,8-sialyltransferase such as the enzyme encoded by the *Campylobacter jejuni* cst-II gene.

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### C. Glyco-Conjugation of Small Molecules

#### 27. Synthesis of CMP-SA-Levulinic acid

This example sets forth the procedure for the synthesis of CMP-SA-levulinic acid.

**Preparation of 2-levulinamido-2-deoxy-D-mannopyranose.** Isobutylchloroformate

(100  $\mu$ L, 0.77 mmol) was added dropwise to a solution of levulinic acid (86  $\mu$ L, 0.84 mmol), anhydrous THF (3 mL) and triethylamine (127  $\mu$ L, 0.91 mmol). This solution was stirred for 3 hours at room temperature and was then added dropwise to a solution containing D-mannosamine hydrochloride (151 mg, 0.7 mmol), triethylamine (127  $\mu$ L, 0.91 mmol), THF (2 mL) and water (2 mL). The reaction mixture was stirred 15 hours and then concentrated to dryness by rotary evaporation. Chromatography (silica, step gradient of 5-15% MeOH/CH<sub>2</sub>Cl<sub>2</sub>) was used to isolate the product yielding 0.156 g (73% yield) of a white solid: R<sub>f</sub> = 0.41 (silica, CHCl<sub>3</sub>/MeOH/water 6/4/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  2.23 (s, 3H), 2.24 (s, 3H), 2.57 (td, J = 6.54, 3.68, 2H) 2.63 (t, J=6.71, 2H), 2.86-2.90 (m, 4H), 3.42 (m, 1H), 3.53 (t, J=9.76, 1H), 3.64 (t, J=9.43, 1H), 3.80-3.91 (m, 4H), 4.04 (dd, J = 9.79, 4.71, 1 H), 4.31 (dd, J = 4.63, 1.14, 1H), 4.45 (dd, J=4.16, 1.13, 1H), 5.02 (d, J=1.29, 1H), 5.11(s, J=1.30, 1H), MS (ES); calculated for C<sub>11</sub>H<sub>19</sub>NO<sub>7</sub>, 277.27; found [M+1] 277.9.

**Preparation of 5-levulinamido-3,5-dideoxy-D-glycero-D-galacto-2-**

**nonulopyranosuronate.** Sodium pyruvate (0.616 g, 5.6 mmol) and N-acetylneuraminc acid aldolase (50 U) was added to a solution of 2-levulinamido-2-deoxy-D-mannopyranose (0.156 g, 0.56 mmol) in 0.1 M HEPES (pH 7.5). The reaction mixture was heated to 37 °C for 20

hours and after freezing. The reaction mixture was then filtered through C18 silica, frozen and freeze-dried. The crude solid was purified using flash chromatography (silica, first using 10-40% MeOH/CH<sub>2</sub>Cl<sub>2</sub> and then CH<sub>2</sub>Cl<sub>2</sub>/MeOH/H<sub>2</sub>O 6/4/0.5). Appropriate fractions were combined and concentrated yielding 45 mg (80% yield) of a white solid: R<sub>f</sub> = 0.15 (silica, CHCl<sub>3</sub>/MeOH/water 6/4/1); <sup>1</sup>H NMR (D<sub>2</sub>O, 500 MHz)  $\delta$  1.82 (t, J=11.9, 1H), 2.21 (dd, J = 13.76, 4.84, 1H), 2.23 (s, 3H), 2.57 (app q, J = 6.6, 2H), 2.86-2.95 (m, 2H), 3.15-3.18 (m, 1H),

3.28-3.61 (complex, 1H), 3.60 (dd, J = 11.91, 6.66, 1H), 3.75 (td, J = 6.65, 2.62, 1H), 3.84 (dd, J = 11.89, 2.65, 1H), 3.88-4.01 (complex, 2H), 4.04 (td, J = 11.18, 4.67, 1H), MS (ES); calculated for C<sub>14</sub>H<sub>23</sub>NO<sub>10</sub>, 365.33; found ([M-1]), 363.97.

**Preparation of cytidine-5'-monophosphoryl-(5-levulinamido-3,5-dideoxy- $\beta$ -D-**

**5 glycero-D-galacto-2-nonulopyranosuronate).** 5-Levulinamido-3,5-dideoxy-D-glycero-D-galacto-2-nonulopyranosuronate (50 mg, 137  $\mu$ mol) was dissolved in 2 mL of 100 mM HEPES pH 7.5 buffer and 1 M MnCl<sub>2</sub> (300  $\mu$ L, 300  $\mu$ mol) was added. CTP-2Na<sup>+</sup> (79 mg, 1.5  $\mu$ mol) was dissolved in 5 mL HEPES buffer and was added to the sugar. The sialyltransferase/CMP-neuraminic acid synthetase fusion enzyme (11 U) was added and the reaction mixture stirred at room temperature for 45 hours. The reaction mixture was filtered through a 10,000 MWCO filter and the filtrate, which contained the product of the reaction, 10 was used directly without further purification: R<sub>f</sub> = 0.35 (silica, IPA/water/NH<sub>4</sub>OH 7/2/1).

**28. Glucocerebrosidase-mannose-6-phosphate produced in CHO cells**

**15** This example sets forth the procedure to glycoconjugate mannose-6-phosphate to a peptide produced in CHO cells such as glucocerebrosidase.

**Preparation of asialo-glucoceramidase.** Glucocerebrosidase produced in CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, and is incubated with 300 mU/mL sialidase-agarose conjugate for 16 hours at 32 °C. To monitor 20 the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel and SDS-PAGE performed according to Invitrogen procedures. The mixture is centrifuged at 10,000 rpm and the supernatant is collected. The beads are washed 3 times with Tris-EDTA buffer, once with 0.4 mL Tris-EDTA buffer, and once with 0.2 mL of the Tris-EDTA buffer. All supernatants are pooled. The supernatant is dialyzed at 4 °C against 50 mM Tris-HCl pH 25 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>, and then twice more against 50 mM Tris-HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated using a Centricon Plus 20 centrifugal filter. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**30 Preparation of Glucocerebrosidase-SA-linker-Mannose-6-phosphate (procedure**

**1).** Asialo-glucocerebrosidase from above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl,

0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-linker-Man-6-phosphate and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of sialic acid-linker-Man-6-phosphate, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas TSK-Gel-3000 analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Glucocerebrosidase-SA-linker-Mannose-6-phosphate (procedure**

2). Glucocerebrosidase, produced in CHO but incompletely sialylated, is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-linker-Man-6-phosphate and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of sialic acid-linker-Man-6-phosphate, a small aliquot of the reaction had CMP-SA-PEG-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas TSK-Gel-3000 analytical column using PBS buffer (pH 7.1). The fluorescent label incorporation into the peptide is quantitated using an in-line fluorescent detector. When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**29. Glycoconjugation of mithramycin to an antibody**

This example sets forth the procedures to glycoconjugate a small molecule, such as mithramycin to Fc region glycans of an antibody molecule produced in mammalian cells. Here, the antibody Herceptin™ is used, but one of skill in the art will appreciate that the method can be used with many other antibodies.

Preparation of Herceptin™-Gal-linker-mithramycin. Herceptin™ is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM UDP-galactose-linker-mithramycin and 0.1 U/mL of galactosyltransferase at 32°C for 2 days to introduce the mithramycin in the Fc region 5 glycans. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The fractions containing product are combined, concentrated, buffer exchanged and then freeze-dried. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples 15 are dialyzed against water and analyzed by MALDI-TOF MS.

### 30. Glycoconjugation of geldanamycin to an antibody

This example sets forth the glycoconjugation of a small molecule, such as geldanamycin, to the Fc region glycans of an antibody produced in CHO cells, such as 20 Rituxan™. Here, the antibody Rituxan™ is used, but one of skill in the art will appreciate that the method can be used with many other antibodies.

Preparation of Rituxan™-Gal-linker-geldanamycin. Rituxan™ is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM UDP-galactose-linker-geldanamycin and 0.1 U/mL of 25 galactosyltransferase at 32°C for 2 days to introduce the geldanamycin in the Fc region glycans. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label incorporation into the peptide is quantitated using an in-line 30 radiation detector.

When the reaction is complete, the reaction mixture is purified using a Toso Haas TSK-Gel-3000 preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The fractions containing product are combined, concentrated, buffer exchanged and then freeze-dried. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**D. Glyco-conjugation of peptides**

**31. Transferrin-GDNF**

This example sets forth the procedures for the glycoconjugation of proteins, and in particular, transferrin is glycoconjugated to GDNF. Transferrin-SA-Linker-Gal-UDP is prepared from transferrin. The galactose residue is removed from GNDNF glycans, and Transferrin-SA-Linker-Gal-UDP is conjugated to GNDNF glycans using a galactosyltransferase.

**Preparation of agalacto-GDNF.** GDNF produced in NSO cells (NSO murine myeloma cells) is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, and is incubated with 300 mU/mL beta-galactosidase-agarose conjugate for 16 hours at 32°C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel performed according to Invitrogen procedures. The mixture is centrifuged at 10,000 rpm and the supernatant is collected. The supernatant is dialyzed at 4 °C against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice more against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated using a Centricon Plus 20 centrifugal filter and stored at -20 °C. The conditions for the IEF gel are run according to the procedures and reagents provided by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Transferrin-SA-Linker-Gal-UDP.** Asialo-transferrin is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with CMP-sialic acid-linker-Gal-UDP (molar amount to add 1 molar equivalent of nucleotide sugar to transferrin) and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of sialic acid, a small aliquot of the reaction has <sup>14</sup>C-SA-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso

Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

The solution is incubated with 5 mM CMP-*sialic acid* and 0.1 U/mL of ST3Gal3 (to cap any unreacted transferrin glycans) at 32°C for 2 days. The incorporation into the peptide is quantitated using an in-line UV detector. After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

10        **Preparation of Transferrin-SA-Linker-Gal-GDNF.** The transferrin-SA-Linker-Gal-UDP prepared as described above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 2.5 mg/mL agalacto-GDNF and 0.1 U/mL of galactosyltransferase at 32°C for 2 days. To monitor the incorporation of galactose, a small aliquot of the reaction has <sup>14</sup>C-galactose-UDP ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). The radioactive label incorporation into the peptide is quantitated using an in-line radiation detector.

When the reaction is complete, the solution is incubated with 5 mM UDP-Gal and 0.1 U/mL of galactosyltransferase (to cap any unreacted transferrin glycans) at 32°C for 2 days followed by addition of 5 mM CMP-SA and 0.1 U/mL of ST3Gal3. After 2 additional days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF

25        MS.

### 32. Glucocerebrosidase-transferrin

This example sets forth the procedures for the glycoconjugation of proteins, and in particular, transferrin is glycoconjugated to glucocerebrosidase. The GlcNAc-ASN structures are created on glucosaminidase, and Transferrin-SA-Linker-Gal-UDP is conjugated to GNDF GlcNAc-ASN structures using galactosyltransferase.

Preparation of GlcNAc-glucocerebrosidase (Cerezyme™). Cerezyme™ (glucocerebrosidase) produced in CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, and is incubated with 300 mU/mL Endo-H-agarose conjugate for 16 hours at 32 °C. To monitor the reaction a small aliquot of the reaction is diluted with  
5 the appropriate buffer and a IEF gel and SDS-PAGE performed according to Invitrogen procedures. The mixture is centrifuged at 10,000 rpm and the supernatant is collected. The beads are washed 3 times with Tris-EDTA buffer, once with 0.4 mL Tris-EDTA buffer and once with 0.2 mL of the Tris-EDTA buffer and all supernatants are pooled. The supernatant is dialyzed at 4 °C against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub> and then twice  
10 more against 50 mM Tris -HCl pH 7.4, 1 M NaCl, 0.05% NaN<sub>3</sub>. The dialyzed solution is then concentrated using a Centricon Plus 20 centrifugal filter. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

15 Preparation of Transferrin-SA-Linker-Gal-glucocerebrosidase. Transferrin-SA-Linker-Gal-UDP from above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 5 mM MnCl<sub>2</sub>, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 2.5 mg/mL GlcNAc-glucocerebrosidase and 0.1 U/mL of galactosyltransferase at 32°C for 2 days. To monitor the incorporation of glucocerebrosidase, the peptide is separated by gel filtration on a Toso Haas  
20 G3000SW analytical column using PBS buffer (pH 7.1) and the product detected by UV absorption. The reaction mixture is then purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by  
25 MALDI-TOF MS.

### 33. EPO-Transferrin

This example sets forth the procedures for the glycoconjugation of proteins to O-linked glycans, and in particular, transferrin is glycoconjugated to EPO. The sialic acid  
30 residue is removed from O-linked glycan of EPO, and EPO-SA-linker-SA-CMP is prepared. EPO-SA-linker-SA-CMP is glycoconjugated to asialotransferrin with ST3Gal3.

**Preparation of O-linked asialo-EPO.** EPO (erythropoietin) produced in CHO cells is dissolved at 2.5 mg/mL in 50 mM Tris 50 mM Tris-HCl pH 7.4, 0.15 M NaCl, and is incubated with 300 mU/mL sialidase (*Vibrio cholera*)-agarose conjugate for 16 hours at 32 °C. To monitor the reaction a small aliquot of the reaction is diluted with the appropriate buffer and a IEF gel performed according to Invitrogen procedures. The mixture is centrifuged at 10,000 rpm and the supernatant is collected. The supernatant is concentrated to a EPO concentration of about 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 5 mM CMP-sialic acid and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of sialic acid, a small aliquot of the reaction had CMP-SA-fluorescent ligand added; the label incorporated into the peptide is separated from the free label by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1). When the reaction is complete, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of EPO-SA-linker-SA-CMP.** The O-linked asialo-EPO 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 1 mM CMP-sialic acid-linker-SA-CMP and 0.1 U/mL of ST3Gal1 at 32°C for 2 days. To monitor the incorporation of sialic acid-linker-SA-CMP, the peptide is separated by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1).

After 2 days, the reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) and collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

**Preparation of Transferrin-SA-Linker-SA-EPO.** EPO-SA-Linker-SA-CMP from above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 2.5 mg/mL asialo-transferrin and 0.1 U/mL of ST3Gal3 at 32°C for 2 days. To monitor the incorporation of transferrin, the peptide is separated by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1) and the

product detected by UV absorption. When the reaction is complete, the solution is incubated with 5 mM CMP-SA and 0.1 U/mL of ST3Gal3 (to cap any unreacted transferrin glycans) at 32°C for 2 days. The reaction mixture is purified using a Toso Haas G3000SW preparative column using PBS buffer (pH 7.1) collecting fractions based on UV absorption. The product  
5 of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

#### 34. EPO-GDNF

10 This example sets forth the procedures for the glycoconjugation of proteins, and in particular, the preparation of EPO-SA-Linker-SA-GDNF.

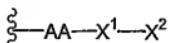
Preparation of EPO-SA-Linker-SA-GDNF. EPO-SA-Linker-SA-CMP from above is dissolved at 2.5 mg/mL in 50 mM Tris-HCl, 0.15 M NaCl, 0.05% NaN<sub>3</sub>, pH 7.2. The solution is incubated with 2.5 mg/mL GDNF (produced in NSO) and 0.1 U/mL of ST3Gal3  
15 at 32°C for 2 days. To monitor the incorporation of GDNF, the peptide is separated by gel filtration on a Toso Haas G3000SW analytical column using PBS buffer (pH 7.1) and the product detected by UV absorption. When the reaction is complete, the solution is incubated with 5 mM CMP-SA and 0.1 U/mL of ST3Gal3 (to cap any unreacted GDNF glycans) at 32°C for 2 days. The reaction mixture is purified using a Toso Haas G3000SW preparative  
20 column using PBS buffer (pH 7.1) collecting fractions based on UV absorption. The product of the reaction is analyzed using SDS-PAGE and IEF analysis according to the procedures and reagents supplied by Invitrogen. Samples are dialyzed against water and analyzed by MALDI-TOF MS.

25 The disclosures of each and every patent, patent application, and publication cited herein are hereby incorporated herein by reference in their entirety.

While this invention has been disclosed with reference to specific embodiments, it is apparent that other embodiments and variations of this invention may be devised by others skilled in the art without departing from the true spirit and scope of the invention. The  
30 appended claims are intended to be construed to include all such embodiments and equivalent variations.

What is claimed is:

1. A cell-free, in vitro method of remodeling a peptide having the formula:



wherein

5 AA is a terminal or internal amino acid residue of said peptide;

X<sup>1</sup>-X<sup>2</sup> is a saccharide covalently linked to said AA, wherein

X<sup>1</sup> is a first glycosyl residue; and

X<sup>2</sup> is a second glycosyl residue covalently linked to X<sup>1</sup>, wherein X<sup>1</sup> and X<sup>2</sup> are selected from monosaccharyl and oligosaccharyl residues;

10 said method comprising:

(a) removing X<sup>2</sup> or a saccharyl subunit thereof from said peptide, thereby forming a truncated glycan; and

(b) contacting said truncated glycan with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer said at 15 least one glycosyl donor to said truncated glycan, thereby remodeling said peptide.

2. The method of claim 1, further comprising:

(c) removing X<sup>1</sup>, thereby exposing said AA; and

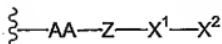
(d) contacting said AA with at least one glycosyltransferase and at least one 20 glycosyl donor under conditions suitable to transfer said at least one glycosyl donor to said AA, thereby remodeling said peptide.

3. The method of claim 1, further comprising:

(e) prior to step (b), removing a group added to said saccharide during post-translational modification.

4. The method of claim 3, wherein said group is a member selected from phosphate, sulfate, carboxylate and esters thereof.

5. The method of claim 1, wherein said peptide has the formula:



wherein

Z is a member selected from O, S, NH, and a crosslinker.

6. The method of claim 1, wherein at least one of said glycosyl donors comprises a modifying group.

7. The method of claim 1, wherein said modifying group is a member selected  
10 from the group consisting of a water-soluble polymer, a therapeutic moiety, a detectable label, a reactive linker group, and a targeting moiety.

8. The method of claim 7, wherein said modifying group is a water soluble polymer.

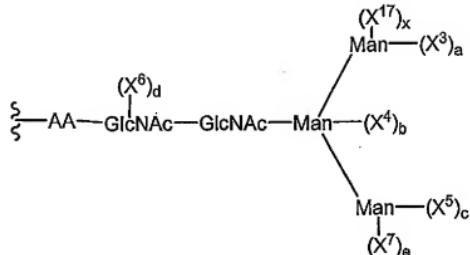
9. The method of claim 8, wherein said water soluble polymer comprises  
15 poly(ethylene glycol).

10. The method of claim 9, wherein said poly(ethylene glycol) has a molecular weight distribution that is essentially homodisperse.

11. The method of claim 1, wherein said peptide is selected from the group consisting of granulocyte colony stimulating factor, interferon-alpha, interferon-beta, Factor  
20 VIIa, Factor IX, follicle stimulating hormone, erythropoietin, granulocyte macrophage colony stimulating factor, interferon-gamma, alpha-1-protease inhibitor, beta-glucosidase, tissue plasminogen activator protein, interleukin-2, Factor VIII, chimeric tumor necrosis factor receptor, urokinase, chimeric anti-glycoprotein IIb/IIIa antibody, chimeric anti-HER2 antibody, chimeric anti-respiratory syncytial virus antibody, chimeric anti-CD20 antibody,

DNase, chimeric anti-tumor necrosis factor antibody, human insulin, hepatitis B sAg, and human growth hormone.

12. A cell-free in vitro method of remodeling a peptide having the formula:



5

wherein

$X^3$ ,  $X^4$ ,  $X^5$ ,  $X^6$ ,  $X^7$  and  $X^{17}$  are independently selected monosacchararyl or oligosacchararyl residues; and

a, b, c, d, e, and x are independently selected from the integers 0, 1 and 2, with the proviso that at least one member selected from a, b, c, d, e, and x is 10  
1 or 2; said method comprising:

- (a) removing at least one of  $X^2$ ,  $X^4$ ,  $X^5$ ,  $X^6$ ,  $X^7$  or  $X^{17}$ , or a saccharyl subunit thereof from said peptide, thereby forming a truncated glycan; and
- (b) contacting said truncated glycan with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer said at 15  
least one glycosyl donor to said truncated glycan, thereby remodeling said peptide.

13. The method of claim 12, wherein said removing of step (a) produces a truncated glycan in which a, b, c, e and x are each 0.

14. The method of claim 12, wherein  $X^3$ ,  $X^5$  and  $X^7$  are selected from the group consisting of  $(mannose)_z$  and  $(mannose)_z(X^8)_y$

wherein

$X^8$  is a glycosyl moiety selected from mono- and oligo-saccharides;

5        $y$  is an integer selected from 0 and 1; and

$z$  is an integer between 1 and 20, wherein

when  $z$  is 3 or greater,  $(mannose)_z$  is selected from linear and branched structures.

15. The method of claim 12, wherein  $X^4$  is selected from the group consisting

10      of GlcNAc and xylose.

16. The method of claim 12, wherein  $X^3$ ,  $X^5$  and  $X^7$  are  $(mannose)_u$ , wherein  $u$  is selected from the integers between 1 and 20, and when  $u$  is 3 or greater,  $(mannose)_u$  is selected from linear and branched structures.

17. The method of claim 12, wherein at least one of said glycosyl donors  
15      comprises a modifying group.

18. The method of claim 17, wherein said modifying group is a member selected from the group consisting of a water-soluble polymer, a therapeutic moiety, a detectable label, a reactive linker group, and a targeting moiety.

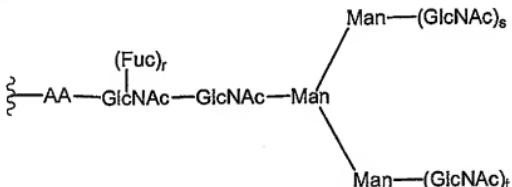
19. The method of claim 18 wherein said modifying group is a water soluble  
20      polymer.

20. The method of claim 19, wherein said water soluble polymer comprises poly(ethylene glycol).

21. The method of claim 20, wherein said poly(ethylene glycol) has a molecular weight distribution that is essentially homodisperse.

22. The method of claim 12, wherein said peptide is selected from the group consisting of granulocyte colony stimulating factor, interferon-alpha, interferon-beta, Factor VIIa, Factor IX, follicle stimulating hormone, erythropoietin, granulocyte macrophage colony stimulating factor, interferon-gamma, alpha-1-protease inhibitor, beta-glucosidase, tissue plasminogen activator protein, interleukin-2, Factor VIII, chimeric tumor necrosis factor receptor, urokinase, chimeric anti-glycoprotein IIb/IIIa antibody, chimeric anti-HER2 antibody, chimeric anti-respiratory syncytial virus antibody, chimeric anti-CD20 antibody, DNase, chimeric anti-tumor necrosis factor antibody, human insulin, hepatitis B sAg, and human growth hormone.

10 23. A cell-free in vitro method of remodeling a peptide comprising a glycan having the formula:



wherein

r, s, and t are integers independently selected from 0 and 1,

15 said method comprising:

- (a) contacting said peptide with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer said at least one glycosyl donor to said glycan, thereby remodeling said peptide.

24. The method of claim 23, wherein at least one of said glycosyl donors  
20 comprises a modifying group.

25. The method of claim 23, wherein said modifying group is a member selected from the group consisting of a water-soluble polymer, a therapeutic moiety, a detectable label, a reactive linker group, and a targeting moiety.

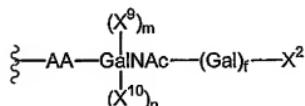
26. The method of claim 25 wherein said modifying group is a water soluble polymer.

27. The method of claim 26, wherein said water soluble polymer comprises poly(ethylene glycol).

5 28. The method of claim 27, wherein said poly(ethylene glycol) has a molecular weight distribution that is essentially homodisperse.

10 29. The method of claim 23, wherein said peptide is selected from the group consisting of granulocyte colony stimulating factor, interferon-alpha, interferon-beta, Factor VIIa, Factor IX, follicle stimulating hormone, erythropoietin, granulocyte macrophage colony stimulating factor, interferon-gamma, alpha-1-protease inhibitor, beta-glucosidase, tissue plasminogen activator protein, interleukin-2, Factor VIII, chimeric tumor necrosis factor receptor, urokinase, chimeric anti-glycoprotein IIb/IIIa antibody, chimeric anti-HER2 antibody, chimeric anti-respiratory syncytial virus antibody, chimeric anti-CD20 antibody, DNase, chimeric anti-tumor necrosis factor antibody, human insulin, hepatitis B sAg, and 15 human growth hormone.

30. The method of claim 1, wherein said peptide has the formula:

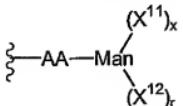


wherein

20  $\text{X}^9$  and  $\text{X}^{10}$  are independently selected monosaccharyl or oligosaccharyl residues; and

$\text{m}$ ,  $\text{n}$  and  $f$  are integers selected from 0 and 1.

31. The method of claim 1, wherein said peptide has the formula:



wherein

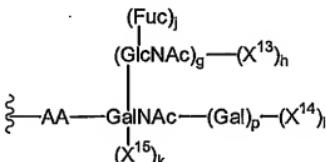
$X^{11}$  and  $X^{12}$  are independently selected glycosyl moieties; and

$r$  and  $x$  are integers independently selected from 0 and 1.

5

32. The method of claim 31, wherein  $X^{11}$  and  $X^{12}$  are  $(\text{mannose})_q$ , wherein  $q$  is selected from the integers between 1 and 20, and when  $q$  is three or greater,  $(\text{mannose})_q$  is selected from linear and branched structures.

33. The method of claim 30, wherein said peptide has the formula:



wherein

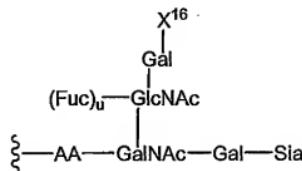
$X^{13}$ ,  $X^{14}$ , and  $X^{15}$  are independently selected glycosyl residues; and g, h, i, j, k, and p are independently selected from the integers 0 and 1, with the proviso that at least one of g, h, i, k and p is 1.

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34. The method of claim 33, wherein

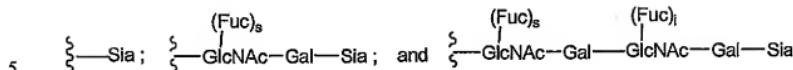
$X^{14}$  and  $X^{15}$  are members independently selected from GlcNAc and Sia; and  $i$  and  $k$  are independently selected from the integers 0 and 1, with the proviso that at least one of  $i$  and  $k$  is 1, and if  $k$  is 1, e.g.,  $h$ , and  $i$  are 0.

35. The method of claim 1, wherein said peptide has the formula:



wherein

$X^{16}$  is a member selected from:

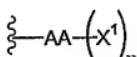


wherein

s, u and i are independently selected from the integers 0 and 1.

36. The method of claim 1, wherein said removing utilizes a glycosidase.

37. A cell-free, in vitro method of remodeling a peptide having the formula:



10

wherein

AA is a terminal or internal amino acid residue of said peptide;

$X^1$  is a glycosyl residue covalently linked to said AA, selected from monosaccharyl and oligosaccharyl residues; and

15

u is an integer selected from 0 and 1,

said method comprising:

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contacting said peptide with at least one glycosyltransferase and at least one glycosyl donor under conditions suitable to transfer said at least one glycosyl donor to said truncated glycan, wherein said glycosyl donor comprises a modifying group, thereby remodeling said peptide.

38. The method of claim 37, wherein said modifying group is a member selected from the group consisting of a water-soluble polymer, a therapeutic moiety, a detectable label, a reactive linker group, and a targeting moiety.

39. The method of claim 38 wherein said modifying group is a water soluble  
5 polymer.

40. The method of claim 39, wherein said water soluble polymer comprises poly(ethylene glycol).

41. The method of claim 40, wherein said poly(ethylene glycol) has a molecular weight distribution that is essentially homodisperse.

10 42. The method of claim 37, wherein said peptide is selected from the group consisting of granulocyte colony stimulating factor, interferon-alpha, interferon-beta, Factor VIIa, Factor IX, follicle stimulating hormone, erythropoietin, granulocyte macrophage colony stimulating factor, interferon-gamma, alpha-1-protease inhibitor, beta-glucosidase, tissue plasminogen activator protein, interleukin-2, Factor VIII, chimeric tumor necrosis factor receptor, urokinase, chimeric anti-glycoprotein IIb/IIIa antibody, chimeric anti-HER2 antibody, chimeric anti-respiratory syncytial virus antibody, chimeric anti-CD20 antibody, DNase, chimeric anti-tumor necrosis factor antibody, human insulin, hepatitis B sAg, and human growth hormone.

20 43. A covalent conjugate between a peptide and a modifying group that alters a property of said peptide, wherein said modifying group is covalently attached to said peptide at a preselected glycosyl or amino acid residue of said peptide via an intact glycosyl linking group.

25 44. The covalent conjugate of claim 43, wherein said modifying group is a member selected from the group consisting of a water-soluble polymer, a therapeutic moiety, a detectable label, a reactive linker group, and a targeting moiety.